

EXTRACELLULAR ATP SIGNALLING PATHWAYS IN DETRUSOR SMOOTH MUSCLE

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By

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Abstract

Two neurotransmitters, ACh and ATP, initiate nerve-mediated contractions in detrusor smooth muscle from guinea-pigs and humans with bladder over-activity; whereas only ACh has a role in muscle from stable human bladders. Both Acetylcholine and ATP are rapidly broken down, by acetylcholinesterases and ectonucleotidases respectively. The activity of ectonucleotidase has been shown to be reduced in the unstable human detrusor compared to stable. The reduced activity may contribute to the development of the purinergic component of the unstable human detrusor contraction.

In vitro muscle strip experiments showed that apyrase significantly reduced the force of EFS contractions in guinea pig and over active human detrusor. Tension in guinea-pig was reduced to 81.5 ± 4.5 % of control and by 87.5 ± 7.5 % in overactive human. Apyrase had no effect in stable human detrusor. In guinea-pig detrusor the ectonucleotidase inhibitor ARL 67156 significantly increased the force of EFS contractions.

The ectonucleotidase activity was investigated by measuring the degradation of a number of concentrations of ATP by detrusor samples. The ATP degradation rate in guinea pig and human detrusor was significantly reduced by the presence of ARL 67156. The ARL 67156 sensitive fraction which represented the ectonucleotidase activity solely was not significantly different between the stable and overactive detrusor groups.

RNA extracted from human detrusor samples was used in gene expression assays to investigate the presence of four members of the ectonucleotidase family. ENTPDase 1, 2, 3, and 5 was present in all 18 human detrusor samples. There was significantly less ENTPDase 1 and 3 in over active detrusor samples compared to those that were stable. There was no difference in the levels of ENTPDase 2 and 5 between the two detrusor groups.

Novel ATP sensitive biosensors were used to measure the real time release of ATP from guinea pig detrusor over the frequency range 1-20 Hz. The ATP signal reached a maximal response at a lower frequency compared to tension. The ATP signal $k_{1/2}$, 4.41 ± 3.02 HZ, was significantly lower than that of the tension generated, 12.0 ± 2.59 Hz. In the presence of $1 \mu\text{M}$ atropine the frequency dependence of the ATP signal and tension is comparable. There was no significant difference between the $k_{1/2}$ of the ATP signal, 7.29 ± 3.5 Hz, and that of tension, 3.12 ± 1.6 Hz.

The apyrase experiments show a correlation between the effect of apyrase on nerve-mediated contractions in human and guinea-pig detrusor and the proportion of atropine resistance. This is consistent with the hypothesis that ATP contributes to nerve-mediated contractions in guinea-pig and over-active human bladders by incomplete breakdown prior to its activation of detrusor smooth muscle. This was corroborated by an enhancement of contraction strength in guinea-pig tissue by ARL 67156, which would have reduced further ATP breakdown. The reduced ATP breakdown may be linked to the difference in the levels of ENTPDase 1 and 3 between stable and overactive detrusor.

The use of ATP sensitive biosensors show it is possible to measure the real time release of ATP from detrusor. The ATP release is frequency dependent and there is evidence to suggest that there is preferential release at lower stimulation frequencies.

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I wish to dedicate this PhD to my auntie Ann and all our Thursday nights.

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List of Abbreviations

TTX	tetrodotoxin
ABMA	α - β -methylene-ATP
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
DMSO	dimethyl sulphoxide
ATP	Adenosine triphosphate
ADP	adenosine 5'-diphosphate
AMP	adenosine monophosphate
UTP	Uridine triphosphate
UDP	Uridine diphosphate
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
CTP	Cystine triphosphate
IDP	Inosine diphosphate
Ach	Acetylcholine
NA	Noradrenaline
NO	Nitric oxide
NPY	neuropeptide Y
VIP	vasoactive polypeptide
ARL 67156	6-N,N-dethyl- β , γ -dibromomethylene-D-ATP
PPADS	Pyridoxal-phosphate-6-azophenyl-2,'4'-disulfonic acid
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
EDTA	ethylene diamine tetra acetic acid
DDT	dichloro-diphenyl-trichloroethane
BSA	bovine serum albumin
Ca^{2+}	Free ionised calcium
$[\text{Ca}^{2+}]$	Concentration of free ionised calcium
$[\text{Ca}^{2+}]_i$	Intracellular concentration of free ionised calcium
Na^{2+}	Free ionised sodium
Mg^{2+}	Free ionised magnesium
K^{+}	Free ionised potassium
cAMP	cyclic adenosine monophosphate
ICS	International Continence Society
OAB	Overactive bladder syndrome
UI	Urinary incontinence
LUT	Lower urinary tract
IC	Interstitial cystitis
CPA	2-chloro-N6-cyclopentyladenosine
SPN	Sacral parasympathetic nucleus
SCG	Sympathetic chain ganglia

ENaC	Epithelial Na ⁺ channels
MLCK	Myosin light chain kinase
MLCP	myosin light chain phosphotase
PKC	protein kinase C
SR	Sarcoplasmic reticulum
PKA	protein kinase A
IP ₃	inositol 1,4,5-triphosphate
E-NTPDase	ectonucleoside triphoshate diphosphohydrolayse
E-NPP	ectonucleoside pyrophosphatase /phosphodiesterase
APCR	apyrase conserved regions
HPLC	High performance liquid chromatography
PCR	Polymerase chain reaction
RT-PCR	Real-time polymerase chain reaction
H ₂ O ₂	Hydrogen peroxide
Ag	Silver
AgCl	Silver chloride
Pt	Platinum
IUPHAR	International union of basic and clinical pharmacology
MuLV	Moloney murine leukaemia virus

1.0 Introduction

The function of the lower urinary tract is both to store and periodically release (void) urine. During the storage phase, as the bladder fills with urine produced by the kidneys (up to 500ml) the bladder pressure (intravesical pressure) must remain relatively low and the outflow resistance high. For optimal emptying the bladder must be capable of force generation to initiate and maintain the flow of urine sufficient to overcome the now reduced outflow resistance. For normal storage and voiding to occur all components of the lower urinary tract including the detrusor, bladder neck, urethral sphincter and pelvic floor have to be coordinated.

1.1 STRUCTURE AND FUNCTION OF THE BLADDER

1.1.1 Structure

The bladder is a hollow organ which varies in shape, size and position. The wall of the bladder is composed of three main layers: the adventitial; the muscle; and the epithelial layers. The structure of the bladder is shown in Figure 1.1.

The serosa is the outermost layer surrounding the bladder and consists mainly of soft connective tissue. It is involved in maintaining the position of the bladder.

The muscle layer consists of the detrusor, the trigone and the bladder neck. The smooth muscle of the detrusor in the dome of the bladder comprises smooth muscle bundles which vary in size and orientation. In humans the bundles can be a few millimetres in diameter and may contain several smaller bundles (Zimmerman, 1996). The bundles form an integrated meshwork which, upon contraction, will cause a reduction in all dimensions of the bladder lumen. The trigone is a triangular region between the ureteric orifices and the internal urinary meatus. It has a number of differences in its musculature compared to the rest of the bladder with smaller muscle bundles and a greater amount of connective tissue.

The internal epithelial layer, known as the urothelium, is a dynamic membrane which provides a barrier between the urine within the bladder lumen and the muscle layer. The urothelium consists of three cell layers, the basal layer attached to a basement membrane, the intermediate layer and the superficial apical layer. The cells within the superficial layer are termed “umbrella cells” as they are large hexagonal cells. The urothelium not only acts as a barrier protecting underlying tissue but also exhibits specialised sensory and signalling properties. Studies have shown that the urothelium has the ability to respond to thermal, mechanical and chemical stimuli and express sensor molecules. These molecules include receptors for bradykinin (Chopra *et al*, 2005), purines (Lee *et al*, 2000), noradrenaline (Birder *et al* 1998), acetylcholine (Beckel *et al*, 2002), amiloride/mechano-sensitive Na⁺ channels and a number of TRP channels (Birder *et al*, 2001). Urothelial cells release a number of chemical mediators for instance NO, acetylcholine, ATP and substance P. For example, it was shown in rabbits that ATP is

released in response to an increase in vesical pressure (Ferguson *et al.*, 1997). Upon release ATP is thought to activate P2X₃ receptors located on afferent nerves which may therefore provide a sensory mechanism to initiate a micturition reflex.

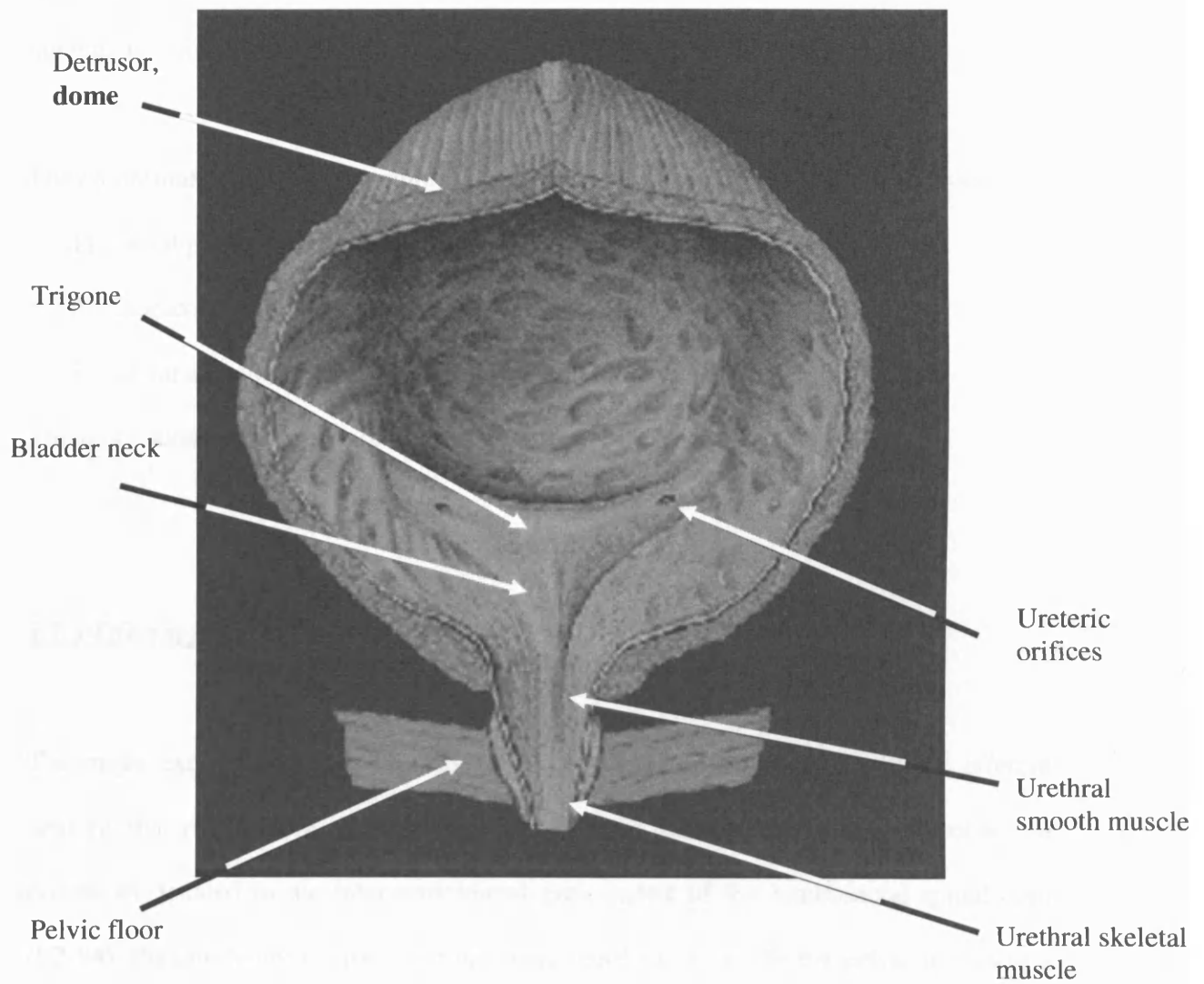


Figure 1.1 The structural components of the lower urinary tract (courtesy of Professor C. Fry)

1.1.2 Innervation of the bladder

Micturition is an autonomic reflex, however release of urine is regulated by voluntary control of neural mechanisms. It involves co-ordination between the bladder and the outlet structures including bladder neck, urethra and sphincter. The bladder must contract raising pressure whilst the outlet relaxes.

The co-ordination of these structures is controlled by three sets of peripheral nerves:

- 1) sacral parasympathetic (pelvic nerves)
- 2) thoracolumbar sympathetic (hypogastric nerves and sympathetic chain)
- 3) sacral somatic nerves (pudendal nerves)

There is a substantial degree of overlap and interaction between these pathways.

1.1.3 Efferent pathways

The major excitatory input to the bladder is provided by the parasympathetic efferent neurons that originate in the sacral parasympathetic nucleus (SPN). The neuronal cell bodies are located in the intermediolateral grey matter of the lumbosacral spinal cord (S2-S4). Preganglionic neurons exit the spinal cord and travel in the pelvic nerves to a pelvic plexus where they synapse with cholinergic postganglionic neurons which innervate the bladder and the urethra. Parasympathetic ganglion cells provide an excitatory input to bladder smooth muscle by release of acetylcholine and ATP and an

inhibitory input to urethral smooth muscle by release of nitric oxide (NO). This results in contraction of the detrusor and relaxation of the urethra during voiding.

The efferent sympathetic pathways arise from the intermediolateral cell column and nucleus intercalatus of the thoracolumbar spinal cord, T₁₁-L₂. The preganglionic neurons pass to the sympathetic chain ganglia (SCG) and then via splanchnic nerves to the paravertebral inferior mesenteric ganglia. Post-ganglionic neurons from the SCG pass to the bladder via the pelvic nerves and the fibres from the inferior mesenteric ganglia travel in the hypogastric nerves (de Groat, 2006). Sympathetic post-ganglionic nerves release noradrenaline to provide an excitatory input to smooth muscle of the urethra and bladder base resulting in contraction via α_1 , and relaxation of detrusor via β_3 -adrenoceptors (Furuta et al, 2006). Sympathetic neurons also cause inhibition and facilitation input to parasympathetic ganglia (de Groat, 1998). The culmination of efferent sympathetic innervation is contraction of the bladder neck and sphincter and at the same time inhibition of detrusor contraction during filling.

The external urethral sphincter (rhabdosphincter) and the pelvic floor muscles are innervated by the efferent somatic pathways. They originate from Onuf's nucleus which is located in the anterior horn cells of the S₂-S₄ sacral spinal cord. The axons exit the spinal cord in the pudendal nerve (Chai and Steers 1996). The sphincter muscles are excited by the release of acetylcholine which stimulate postjunctional nicotinic receptors (de Groat, 2006) resulting in contraction.

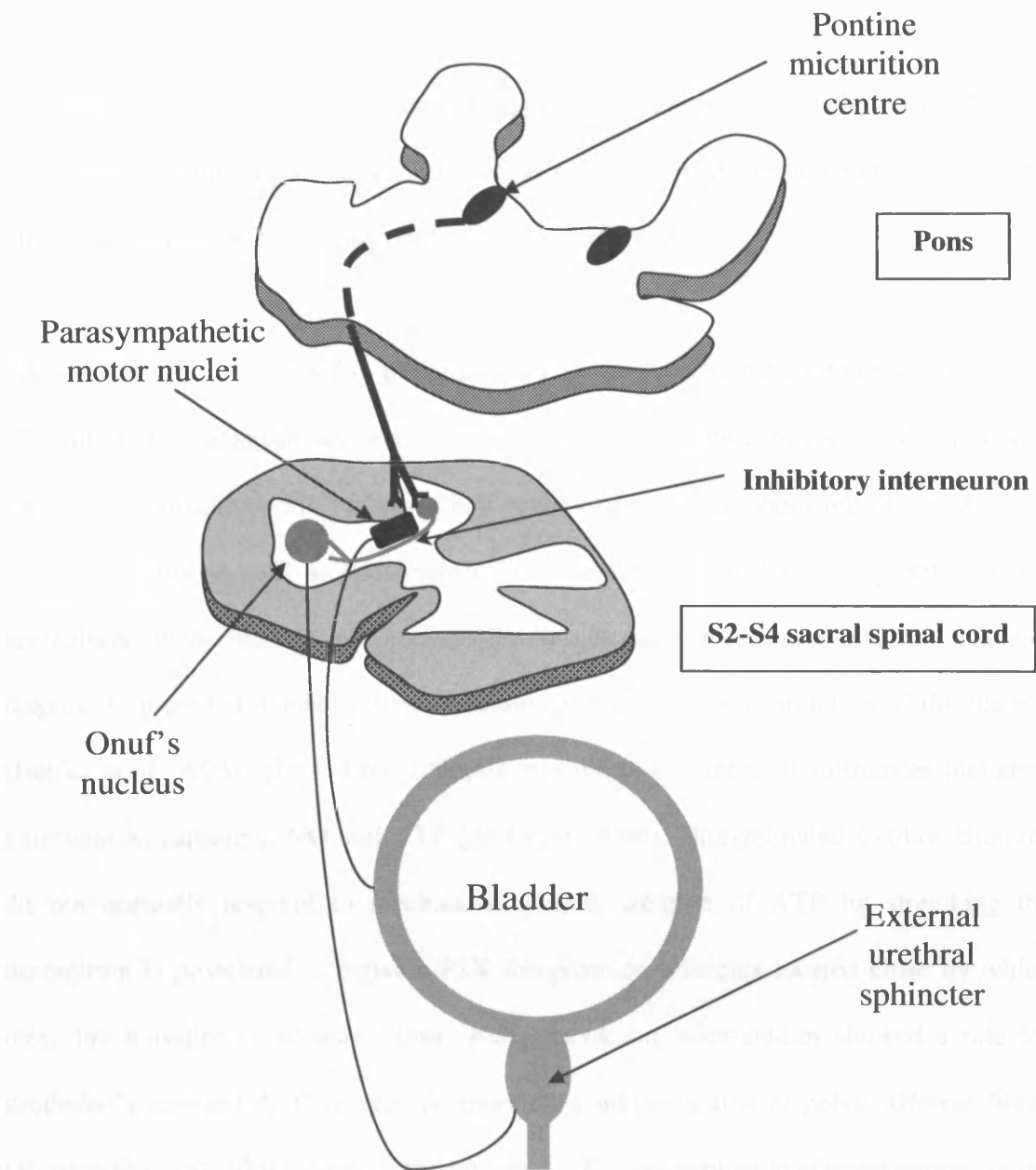


Figure 1.2 Schematic of efferent pathways to the bladder. Signals to void are sent from the pontine micturition centre to inhibit the activity of the somatic pathway to relax the urethral muscle. There is also release of the inhibition on the parasympathetic motor nuclei to increase the contractile activity of the bladder

1.1.4 Afferent Pathways

The afferent pathways provide a sensory input in the control of the bladder and sphincter and provide an ongoing signal during the filling phase. The distension and volume of the bladder as well as amplitude of contraction are monitored.

Afferent activity is conveyed to the central nervous system by three sets of nerves. Those afferent axons passing in the pelvic nerve to the sacral spinal cord are small myelinated (A- δ) and unmyelinated (C) fibres. They convey information about muscle tension and bladder volume as well as nociception (Sugaya, 2005) from the serosa, detrusor and urothelium of the bladder and urethra. Studies in cats have shown that A- δ afferents respond in a graded manner to passive distension and active contraction of the bladder (Häbler *et al*, 1993). The C-fibre afferents respond to a number of substances including neurotoxins, capsaicin, NO and ATP (de Groat, 2006). Unmyelinated C-fibre afferents do not normally respond to mechanical stimuli. Release of ATP by stretching the urothelium is postulated to activate P2X receptors on afferents located close by which relay the sensation of bladder filling. P2X₃ knock-out mice studies showed a role for urothelially-released ATP via the receptors on a subpopulation of pelvic afferent fibres (Vlaskovska *et al*, 2001). Also, in the rat when ATP was applied to afferent nerves on the surface of the bladder the firing induced by bladder distension was enhanced and the threshold for electrical stimulation of A- δ and C-fibre afferents was reduced (Yu & de Groat, 2004).

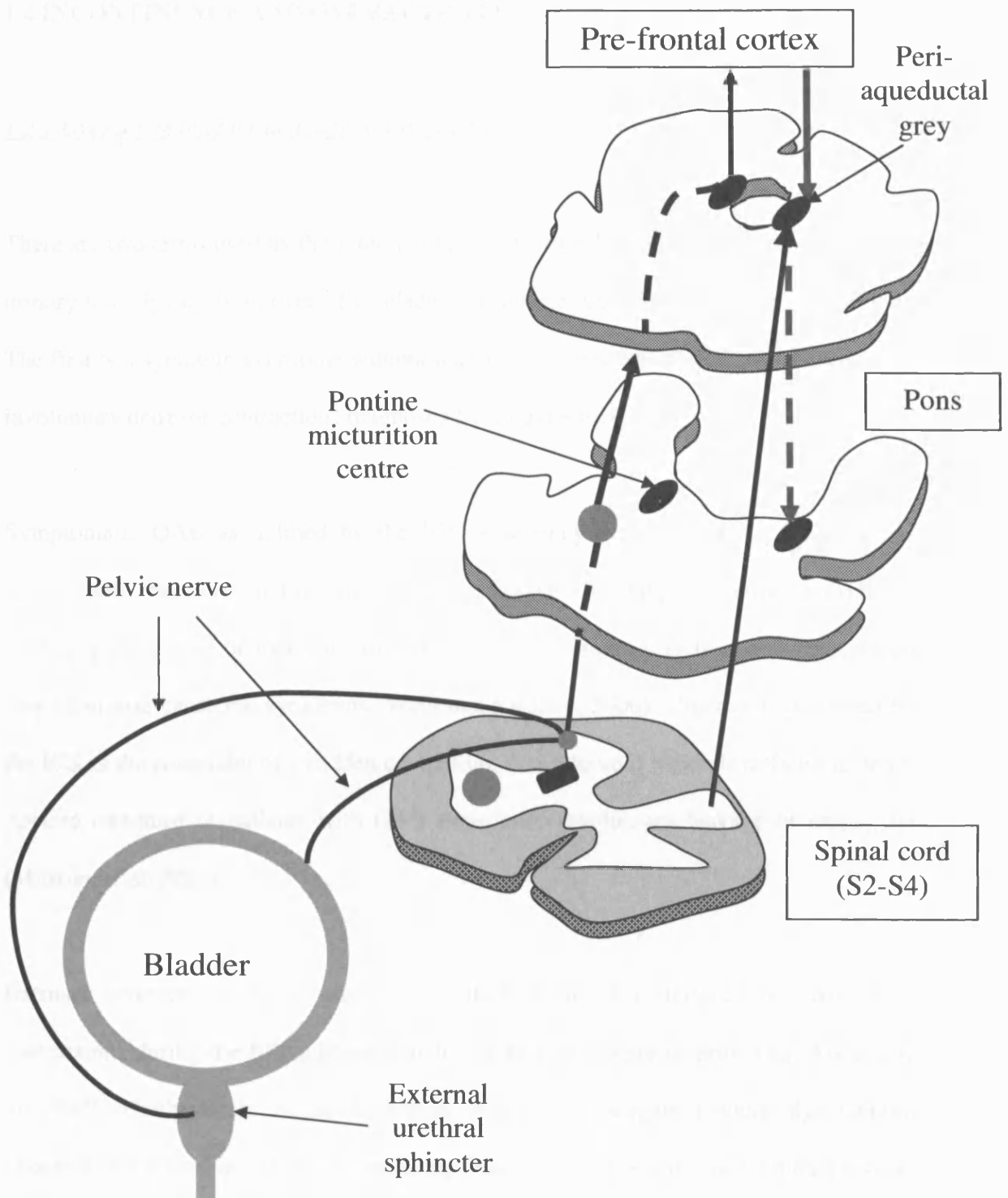


Figure 1.3 Schematic of afferent pathways from the bladder. Afferent fibres run from the bladder to the spinal cord, transmitting the signal to the peri-aqueductal grey and higher centres of the brain

1.2 INCONTINENCE AND OVERACTIVITY

1.2.1 Overactive bladder and detrusor overactivity

There are two terms used by the International Continence Society (ICS) to describe lower urinary tract dysfunction, overactive bladder syndrome (OAB) and detrusor overactivity. The first is a symptom syndrome without a definitive cause and the second describes the involuntary detrusor contractions diagnosed by urodynamics.

Symptomatic OAB as defined by the ICS is urgency with or without incontinence, usually with frequency and nocturia (Abrams, 2003). The symptoms must be exhibited without pathological or metabolic disorders, such as bladder infection or cancer, which may otherwise cause the symptoms (Wein and Rackley, 2006). Urgency is described by the ICS as the complaint of a sudden compelling desire to void which is difficult to defer. Around one-third of patients with OAB experience involuntary leakage of urine (UI) (Milsom, et al, 2001).

Detrusor overactivity is a urodynamic observation characterised by involuntary contractions during the filling phase which may be involuntary or provoked (Abrams *et al* 2002). Urodynamics is the technique used to investigate bladder dysfunction. Observations are made of the bladder during filling, natural or artificial, and the pressure and flow of urine during voiding. This is done by measuring two different pressures, intravesical and abdominal. Intravesical pressure is the pressure within the bladder

measured by insertion of a catheter. Abdominal pressure is measured with a rectal or vaginal catheter, and is the estimated pressure surrounding the bladder. Detrusor pressure is described as the component of intravesicular pressure that is created by the force of the bladder wall. It is obtained by subtracting abdominal pressure from the intravesical pressure. Normal detrusor function is when there is little or no change in pressure associated with filling, with no involuntary contractions. When there are involuntary detrusor contractions that are spontaneous or provoked, detrusor function is considered to be overactive.

Detrusor overactivity can be divided by cause into idiopathic and neurogenic detrusor overactive groups. Neurogenic detrusor overactivity describes patients where there is a relevant neurological condition for example spinal cord injury, multiple sclerosis, Parkinson's disease and stroke. Idiopathic detrusor overactivity is when there is no defined cause.

1.2.2 Prevalence and management

Patients who suffer with bladder overactivity experience a wide range of symptoms including, urgency, frequency, nocturia and incontinence. The incidence of bladder overactivity has been evaluated in studies in both Europe and in the United States. The studies reported similar findings, that prevalence is high within the general population (Wein and Rackley, 2006). The European study indicated that 16.6% of people over 40 had a least one symptom of OAB with 36% experiencing urge incontinence (Milsom, *et*

al, 2001). The incidence of OAB also increases with advancing age, with detrusor overactivity being the most common cause of incontinence in the elderly (Wagg and Cohen, 2002). All symptoms of OAB significantly affect the patients' quality of life, 66% of those with symptoms in the Milsom study reported effects on their daily lives.

There are a number of treatments for bladder overactivity ranging from changes of lifestyle, bladder training, pharmacotherapy and surgery. There are currently three classes of drugs used in treatment; antimuscarinics, antispasmodics and mixed antimuscarinics/antispasmodics (Newgreen, 1998). The most widely used treatment in the UK is antimuscarinics (Wagg and Cohen, 2002), for example oxybutynin, tolterodine and trospium chloride (Hashim and Abrams, 2004). However, there are a number of side-effects associated with these drugs due to the low selectivity for muscarinic receptors in the bladder. The side-effects, including dry mouth, constipation and blurred vision result in the agents' poor tolerance. More recently developed antimuscarinics such as darifenacin and solifenacin succinate are selective M₃ receptor antagonists and may reduce the incidence of dry mouth.

Research into the use of intravesical botulinum toxin as a treatment for spontaneous contractions is ongoing. One action of the toxin is believed to inhibit the exocytosis of synaptic vesicles therefore inhibiting the release of Ach from the neuromuscular junction (Haferkamp *et al*, 2004). Clinical studies have shown the effectiveness of the treatment. Kuo, (2004) reported 73.3% success, with improvements in symptoms, in patients with detrusor overactivity refractory to anticholinergics.

1.2.3 Pathophysiology

The normal function of the bladder during the filling phase is to store increasing volumes of urine whilst maintaining a low intravesical pressure. However in OAB involuntary contractions occur, resulting in uninhabitable rises of pressure. As the function of the LUT involves complex coordination of muscular and nervous activity there are numerous stages at which changes could cause OAB: a number of hypothesis exist as to their cause.

One hypothesis is that the basis for bladder overactivity is myogenic, that changes in the normal compliant detrusor smooth muscle compromise normal function (Turner and Brading, 1997). To be compliant the smooth muscle must be able to stretch and rearrange to enable increasing volumes at low pressures, and also to contract synchronously to cause complete emptying. Outflow obstruction can compromise function as it causes increased intravesical pressure leading to hypertrophy of the bladder and individual detrusor cells, reduced blood flow and partial denervation (Fry and Wu, 1998). Numerous changes have been observed in the detrusor from overactive bladders from humans and animal models, for example some overactive bladders have abnormal spontaneous mechanical activity and some show supersensitivity to muscarinic agonists and high extracellular potassium solution (Brading, 1997). Physical changes to the detrusor have been observed with light and electron microscopy, including increased deposition of elastin and collagen. The theory is that all of these changes lead to increased excitability and increased ability of activity to spread between cells (Brading,

1997) resulting in “micromotions” of the detrusor and rises in intravesical pressure associated with OAB (Wein and Rackly, 2006).

The functions of the lower urinary tract depend on neural circuits located in the brain, spinal cord and peripheral ganglia. Thus, changes or damage to any of these pathways may lead to OAB. Diseases and injuries such as those described on page 10 are all causes of neurogenic OAB. The changes that occur as a result include reduction in peripheral or central inhibition, enhancement of excitatory transmission in the micturition pathway, increased primary afferent input from the lower urinary tract and emergence of bladder reflexes resistant to central inhibition (de Groat, 1997).

A recently developed hypothesis proposed by Gillespie, (2004) is the autonomous bladder hypothesis; that is, OAB is a consequence of inappropriate activation or modulation of phasic activity. Experiments were performed on whole guinea-pig bladders, and showed that during induced phasic activity by α , β -methylene ATP and substance P there was a rise in intravesical pressure and an increased frequency of phasic activity. It was proposed that phasic activity consists of transient rises of intravesical pressure during the filling phase that is not generated through the same mechanisms that activate the detrusor in micturition, implying that there is a second mechanism within the bladder wall.

Ferguson et al, (1997) showed that hydrostatic pressure changes of the rabbit urinary bladder resulted in ATP release from the basal-lateral surface. They proposed that ATP

plays a sensory role in normal micturition, where urothelial-derived ATP stimulates sub urothelial afferents. In some bladder pathologies including interstitial cystitis (Sun et al, 2001; Birder et al, 2003) this stretch activated release of ATP has been shown to be increased. The stretch induced ATP release has also been shown to be increased in ageing bladders (Yoshida et al, 2004). It is proposed that the ATP release is mediated by amiloride sensitive epithelial Na⁺ channels (ENaC) (Du et al, 2007).

Other hypotheses involve the role of the urothelium in sensory functions and release of Ach, both from the urothelium and nerves. The urothelium releases neurotransmitters in response to stretch and other stimuli. Yoshida *et al*, 2004, showed that basal release of Ach from the detrusor was mainly from the urothelium as release was not TTX-sensitive and was significantly reduced when the urothelium was removed. The release of Ach has also been shown to increase with age-related changes (de Groat, 2004) and muscarinic-receptor functions can change in bladder disorders. These factors can alter the excitability of bladder afferent nerves acutely and chronically which may be important in bladder overactivity.

As described above there are a number of hypotheses as to the cause of OAB including myogenic or neurogenic changes, alterations in phasic activity, changes in the role of urothelium and altered release of ATP. This thesis will investigate the role of nerve released ATP in detrusor contraction and the contribution of this to OAB.

1.3 DETRUSOR SMOOTH MUSCLE CONTRACTION

There are a number of properties of detrusor smooth muscle that are important in the normal function of the urinary bladder. As the bladder fills, the myocytes must rearrange and stretch to allow increases in bladder volume and at the same time maintain a low intravesical pressure. During micturition force generation and shortening must be initiated comparatively quickly and contraction needs to be synchronised (Andersson, 2004). Based on the contractile behaviour of detrusor smooth muscle, it is described as phasic smooth muscle and is due to its twitch-type contractions that are initiated by excitatory nerves.

1.3.1 Contractile proteins

As with other muscles, contraction of detrusor smooth muscle is due to interaction of the contractile proteins actin and myosin. There are three types of filament present in smooth muscle cells, thick, intermediate and thin filaments. The thick filaments consist of myosin; two α -helical heavy chains, from the myosin II family, that contain ATP-hydrolytic and actin binding sites (Zimmerman *et al*, 1996, Arner *et al*, 2003) and two pairs of myosin lights chains. The intermediate filaments are composed of desmin and vimentin which may not be directly involved in the contractile process but form the cytoskeleton of the cell, maintaining cell shape and distributing forces (Andersson and Arner, 2004; Zimmermann, 1996). The thin filaments consist of actin monomers of which there are four different isoforms, and tropomyosin which may aid binding of

myosin heads to actin. The ratio of actin to myosin is about 15:1 in smooth muscle, compared to 2:1 in striated muscle (Anderson and Arner, 2004). Contraction of the cell occurs when force is generated through the interaction of the actin and myosin filaments, cross-bridges form between the filaments making them slide past each other in an ATP-driven process.

1.3.2 Contraction and relaxation

Contraction of detrusor smooth muscle cells depends upon an increase in the concentration of intracellular calcium, $[Ca^{2+}]_i$. Upon stimulation increased free Ca^{2+} binds to the calcium-binding protein calmodulin to form a Ca^{2+} -calmodulin complex, which then binds and activates myosin light chain kinase (MLCK). The activated MLCK phosphorylates serine-19 on the 20 kD regulatory subunit light chain found on the myosin head which is necessary for the cross-bridge to bind to actin. The phosphorylated myosin undergoes repeated cycles of actin-mediated ATP hydrolysis (Andersson and Arners, 2004) resulting in cell contraction.

The mechanisms involved in relaxation include a decrease of intracellular Ca^{2+} which would dissociate the Ca^{2+} -calmodulin complex and prevent activation of MLCK. Ca^{2+} levels fall through reuptake into the SR via Ca^{2+}/Mg^{2+} -ATPase pumps, or removal from the cell by secondary active transport via Na^+/Ca^{2+} exchange (Wu and Fry, 2001).

There is also increased activity of the myosin light chain phosphatase (MLCP). Activation of MLCP causes dephosphorylation of the myosin light chain head preventing cross-bridge formation. However, increased cell sensitivity to Ca^{2+} can be induced by the inhibition of MLCP activity, through phosphorylation of the MLCP myosin-binding subunit. Phosphorylation occurs by a Rho-associated kinase which is activated by Rho, a GTPase of the Ras family (Symons, 1996). The smooth muscle specific protein inhibitor, CPI-17, when phosphorylated by protein kinase C (PKC), can also inhibit MLCP activity and increase Ca^{2+} sensitivity (Eto *et al*, 1999).

MLCK can be phosphorylated which decreases its affinity for the Ca^{2+} -calmodulin complex and decreases the Ca^{2+} sensitivity of the contractile system, reducing contraction. The cAMP-dependent kinase, protein kinase A (PKA) phosphorylates MLCK and can explain how cyclic nucleotides can depress contraction in this tissue (Andersson and Arners, 2004).

1.3.3 Calcium stores

The Ca^{2+} that initiates contraction comes from two main sources, the extracellular space via Ca^{2+} channels and intracellular stores, the sarcoplasmic reticulum (SR). Studies using low doses of thapsigargin, an inhibitor of the sarcoplasmic reticulum Ca^{2+} pump, have shown detrusor contraction is dependent on Ca^{2+} release from the SR (Masters *et al*, 1999). Ca^{2+} release from the SR is through activation of inositol trisphosphate receptors

(IP₃) and by Ca²⁺-induced Ca²⁺-release via ryanodine receptors. A mechanism for replenishing the Ca²⁺ store was proposed by Fry *et al*, (2004). Following release of Ca²⁺, the increase in [Ca²⁺]_i was associated with a brief membrane hyperpolarisation, as Ca²⁺-dependent K⁺ channels are activated. This limited further entry of Ca²⁺ by reducing the open-probability of Ca²⁺ channels, and was followed by a transient fall of [Ca²⁺]_i below the resting level. The reduction of [Ca²⁺]_i , via the mechanisms mentioned above, reduced K⁺ channel activity which depolarised the cell. This subsequently increased the Ca²⁺ channel open probability and resulted in an increase in Ca²⁺ entry, thus replenishing Ca²⁺ levels.

1.4 INITIATION OF CONTRACTION BY NEUROTRANSMITTER RELEASE

Parasympathetic nerve stimulation of the bladders of several mammalian species has been shown to be composed of a cholinergic component and a non-adrenergic, non-cholinergic component. The cholinergic component can be demonstrated in animals through enhancement of contraction with anticholinesterases and reduction of contraction with cholinergic blockers such as atropine. In animals there is an atropine-resistant component of contraction, albeit to different extents, 58% of rabbit contraction and 22% of pig contraction is resistant to atropine (Sibley, 1984). Contraction is not effected by adrenergic or nicotinic antagonists. The non-adrenergic, non-cholinergic component of detrusor contraction was first proposed to be the nucleotide ATP by Burnstock in 1972. Evidence has since grown to support this; the response of rat and guinea pig bladder to exogenous ATP mimics that of nerve stimulation and quinidine can block the response of both exogenous and nerve released ATP (Burnstock *et al* 1972). TTX-sensitive nerve stimulated release of ATP was measured in the superfusate surrounding guinea-pig bladder (Burnstock, 1978) and the atropine-sensitive component of contraction could be abolished by desensitising P2X receptors (Hoyle *et al*, 1989). The criteria required for ATP to be a neurotransmitter, including presence in nerve terminals, release during stimulation and enzymatic breakdown, have thus been demonstrated.

Unlike most animal species humans and old world monkeys show no purinergic component to detrusor contraction. A lack of atropine-resistant contraction has been demonstrated in human bladder (Sibley, 1984, Sjögren *et al*, 1985, Palea *et al*, 1993). It

is believed that conditioning of humans and monkeys to micturate only when it is socially acceptable has eliminated the purinergic component of contraction. Other animals may need to void rapidly and as ATP activation of P2X receptors results in a rapid increase in intracellular Ca^{2+} and contraction compared to muscarinic activation they may have retained this mechanism of micturition. However atropine-resistance of nerve mediated-contraction has been observed in human detrusor from patients with various disorders including interstitial cystitis (Palea *et al*, 1993), overactive bladder (Sjögren *et al*, 1985) and overactive and obstructed bladder (Bayliss *et al*, 1999). The degree of atropine-resistance varies for different patients and different disorders, with the atropine-resistant component as large as 35% of the contraction (Sibley, 1984). The atropine resistant component has also been demonstrated to be positively correlated to an increase of age (Yoshida *et al*, 2001).

ATP is co-released from nerves to activate P2X receptors (see section 1.6.2) on the detrusor smooth muscle to initiate contraction. ATP is then rapidly and sequentially degraded in the synapse by ectonucleotidases to adenosine which is taken back up into the neuron for re-synthesis. There are therefore a number of stages at which alterations could occur and enable a purinergic component of detrusor contraction to emerge.

Sympathetic nerves within the bladder release noradrenaline (NA) to activate adrenergic receptors within the bladder particularly in the trigone region and urethra (Andersson, 1993). β_3 -adrenoceptors (Takeda *et al*, 1999) in the smooth muscle when activated by

NA relax the bladder, but α 1-adrenoceptors in the bladder outlet contract the muscle in this region to keep the bladder outlet closed during filling (de Groat et al, 1999). Their combined action thus aids filling and storage. α -adrenoceptors on peripheral nerves have been shown to facilitate the release of Ach and ATP in the rat bladder (de Groat and Yoshimura, 2001).

The role of each of these major transmitter classes; muscarinic (Ach), purinergic (ATP) and adrenergic, will each be discussed in more detail in following sections.

1.5 MUSCARINIC AND ADRENERGIC RECEPTORS AND DETRUSOR CONTRACTION

In many species there are two main neurotransmitters, Acetylcholine and ATP, released from the embedded parasympathetic motor nerves that stimulate detrusor smooth muscle to contract. With human detrusor, contractions are most importantly mediated by cholinergic mechanisms, however a purinergic component has been observed in some bladder pathologies (Bayliss *et al*, 1999).

1.5.1 Muscarinic Receptors

Muscarinic receptors on detrusor smooth muscle are stimulated by Ach released from post-ganglionic parasympathetic motor nerves. Five genes encode separate muscarinic receptor glycoprotein subtypes, m_1 - m_5 , for which most have been pharmacologically characterised¹ (Malcolm *et al*, 1998). Muscarinic receptors have seven transmembrane regions and are G-protein coupled, although the subtypes and second messenger pathways differ. m_1 , m_3 and m_5 subtypes are coupled to $G_{q/11}$ and activate phospholipase C (PLC) to up-regulate IP_3 production, whereas, m_2 and m_4 are coupled to $G_{i/o}$ and inhibit the activity of adenylyl cyclase (Andersson and Yoshida, 2003).

¹ 'm' indicates muscarinic receptors identified by molecular biochemistry, 'M' by pharmacology.

The mRNAs for the five subtypes of muscarinic receptor have been identified in human bladder (Sigala *et al*, 2002), with m_2 and m_3 prevalent. m_2 and m_3 subtypes are present on detrusor smooth muscle cells, the predominant receptor being m_2 . However, pharmacological experiments using subtype-selective agonists and antagonists have shown that contractile responses are attributed to M_3 receptor activation (Schneider *et al*, 2004, Chess-Williams *et al* 2001). The stimulation of M_3 receptors by muscarinic agonists generates inositol trisphosphate (IP_3) and diacylglycerol after PLC activation. IP_3 activates receptors on the SR to release Ca^{2+} and raise the intracellular $[Ca^{2+}]$. A small Ca^{2+} influx may also accompany this process, possibly through L-type Ca^{2+} channels or non-selective cation channels (Inoue and Brading, 1990).

Studies have shown that L-type Ca^{2+} channels are involved in the contractile process following muscarinic stimulation. Nifedipine inhibition of L-type channels attenuated carbachol-induced contractions in rat detrusor (Schneider *et al* 2004a) and human detrusor (Schneider *et al* 2004b), as did diltiazem in rabbit detrusor (Damaser *et al*, 1997). In guinea-pig tissue muscarinic-dependent contractions were reduced by caffeine and nifedipine indicating both intracellular store release of Ca^{2+} and influx through L-type Ca^{2+} channels are required (Hashianti *et al*, 2000). A role for L-type Ca^{2+} channels in replenishing intracellular Ca-stores after muscarinic activation (Fry *et al*, 2004) has been discussed above (section 1.3.3)

The role of M_2 receptors is less clear, but it may be indirectly involved in the contractile process particularly at lower agonist concentration (Hashianti *et al*, 2000). Ach

stimulation of these receptors can attenuate adenylyl cyclase activity, which would reduce the amount of cellular cAMP, opposing relaxation. There is some evidence to suggest that in some disease states M₂ receptors contribute more directly to bladder contraction. For example, Braverman *et al* (1998) have shown that in the denervated rat bladder M₂ receptors contribute to contraction. In obstructed, hypertrophied rat bladders there was an increase in M₂, and a decrease in M₃ receptor density. Similar results showing M₂-mediated contraction of neurogenic human bladder were observed by Pontari *et al* (2004).

Rho-kinase has also been implicated in contraction following muscarinic stimulation both in animal and human bladder by increasing the sensitivity to Ca²⁺ of the contractile machinery. In rabbit detrusor, bethanechol-induced contractions were almost abolished by a combination of Rho-kinase and non-selective cation channel inhibitors (Jezior *et al*, 2001). Rho-kinase inhibitors also reduced carbachol-induced contractions in rat bladder (Fleischmann *et al* 2004) and concentration-dependently attenuated contractions in human bladder (Schneider *et al* 2004b).

Muscarinic receptors are also found presynaptically and can be involved in regulation of neurotransmitter release. Pre-synaptic M₁ receptors are facilitatory and M₄ inhibitory to neuromuscular function. In the rat bladder, continuous electrical field stimulation enhanced the release of Ach via M₁ receptors, using PKC second messenger systems and L- and N-type Ca²⁺ channel function (Somogyi *et al*, 2003). A presynaptic M₁ facilitatory mechanism has also been observed in human detrusor (Tobin and Sjogren,

1995). The inhibitory effects of M_4 receptors were shown in human detrusor where Ach acts as a negative feedback modulator to reduce further Ach release (D'Agostino *et al*, 2000). Experiments have shown that in the spinal cord-transected rat the pre-synaptic M_1 receptor facilitatory mechanism is replaced by a facilitatory M_3 receptor mechanism (Somogyi *et al*, 2003).

Muscarinic receptors are also located on the urothelium, the mRNA for all five subtypes have been identified in human tissue (Tyagi *et al*, 2006). Saturation curves for [3 H]-QNB, a non-specific muscarinic ligand, binding showed a greater muscarinic receptor density in the urothelium compared to the detrusor smooth muscle (Hawthorn *et al*, 2000). The roles of muscarinic receptors in the urothelium are not fully understood. However M_2 receptors have been shown to induce the release of a diffusible factor that is able to reduce contraction of the detrusor (Chess-Williams, 2002).

1.5.2 Adrenergic receptors

There are three major classes of adrenergic receptors, α_1 , α_2 and β – receptors. The classes are further subdivided: α_1 receptors into α_{1A} , α_{1B} , α_{1D} and α_{1I} ; α_2 receptors into α_{2A} ; and α_{2B} and the β receptors are divided into β_1 , β_2 and β_3 . They all have seven transmembrane domains and are G-protein coupled although each group activates different signalling pathways. The α_1 receptors increase phospholipase C activity, α_2

receptors decrease the activity of adenylate cyclase, whilst the β receptors stimulate adenylate cyclase activity leading to elevation of cAMP (Docherty 1998).

Adrenergic receptors are mainly found in the bladder neck and trigone regions of the bladder. In the human bladder there is an overall low expression of the α_1 receptors with only α_{1A} and α_{1D} being expressed with two-thirds being α_{1D} (Malloy *et al*, 1998). The expression is slightly different in the rat as there is greater expression of α_{1A} compared to α_{1D} and very little α_{1B} (Hampel *et al*, 2002). The role of these receptors in the bladder is still to be fully determined. A study showed there is no response from the detrusor with application of concentrations of the α -agonist phenylephrine up to 10 μ M (Nomiya and Yamagauchi, 2003). They also showed that expression levels of α receptors were not changed in patients with obstructed bladders and that obstruction did not significantly alter the response to phenylephrine. However de Groat and Yoshimura (2001) have shown that α_1 activation promoted the closure of the bladder outlet. Furthermore α receptors on peripheral nerves have been demonstrated to facilitate the release of both Ach and ATP in the rat bladder.

All three subtypes of β adrenoceptors are expressed in human and animal detrusor and the predominant subtype is β_3 (Fujimura *et al*, 1999). Isoprenaline, noradrenaline and adrenaline produce a concentration-dependent relaxation of human detrusor (Igawa *et al*, 1998) and the β_3 receptor has been implicated in detrusor relaxation (Yamaguchi, 2002). Activation of this receptor subtype in animal models increases bladder capacity and has been suggested to play a role in the storage phase in human bladder (Takeda *et al*, 1999).

1.6 ATP AND PURINE RECEPTORS IN DETRUSOR SMOOTH MUSCLE

1.6.1 ATP as a neurotransmitter

ATP has long been known as a ubiquitous intracellular source of energy but it was in 1929 that the potent extracellular effects of adenosine nucleotides and adenosine were reported in guinea pig (Drury and Szent-Györgyi, 1929). It was sometime later that Burnstock first posed the questions is ATP a neurotransmitter; and do some nerves release more than one neurotransmitter (Burnstock 1972, Burnstock 1976). Since the seventies evidence has grown to show that co-transmission is the rule rather than the exception (Sneddon *et al* 1996) and that ATP can be co-released within the central and peripheral nervous systems with a number of substances including noradrenaline (NA), acetylcholine (Ach), neuropeptide Y (NPY), nitric oxide (NO), and vasoactive polypeptide (VIP) (Burnstock 1999).

In nerve terminals ATP has been shown to be present individually and co-packaged with Ach and NA in all types of synaptic vesicles (Sperlágh and Vizi 1996; Bodin and Burnstock, 2001; Zimmermann and Whittaker, 1977). Although variable storage ratios have been observed in different systems there is always less ATP than NA or Ach (Sperlágh and Vizi 1996) and it is likely this would be true in the bladder. The neurotransmitter ratios can change and vesicle composition within a nerve terminal may be heterogeneous. Electrical stimulation can affect the yield and composition of synaptic vesicles (Zimmermann and Whittaker, 1974) and cocaine was shown to decrease

preferentially the ATP content of noradrenergic vesicles in rat tail artery (Palaty, 1991). In the bladder the fluorescent marker quinacrine, which complexes with ATP, was positive in numerous intermural neurones in the guinea pig-bladder (Crowe *et al*, 1986). As the composition of synaptic vesicles can change it may be possible that in some bladder pathologies where atropine-resistance is observed the ATP content increases in co-packaged vesicles. Alternatively more individual ATP vesicles could be exocytosed resulting in more prominent ATP-induced contractions.

Functional studies and measurement of neuronal-stimulated overflow of ATP with luciferin-luciferase assays have confirmed the release of ATP with Ach from motor nerves terminals in a number of tissues (Vizi *et al*, 2000; Silinsky and Redman, 1996). In the bladder TTX-sensitive nerve-stimulated release of ATP has also been measured (Burnstock *et al* 1978) and there is some evidence to suggest the ATP is co-released from the same nerves as Ach (MacKenzie *et al*, 1982). In sympathetic nerves differential co-transmission of ATP and NA has been observed suggesting that the two transmitters are from separate populations of exocytotic vesicles (Todorov *et al* 1996). This study, together with a second, showed a biphasic contraction in guinea-pig vas deferens, with ATP mediating the initial twitch phase and NA the tonic phase, showing either release was not simultaneous, or that there were different pathways in contractile activation. The twitch contraction with stimulation up to 8 Hz was mediated by ATP, but above 8 Hz NA contributed to the twitch by enhancing postjunctional actions of ATP (Todorov *et al* 1999). The release of ATP has been shown to be dependent on stimulation frequency in nerves that mediated contraction of other smooth muscles (Silinsky and Redman, 1996).

Co-transmission of ATP and NA in rabbit ear artery showed that ATP action was more prominent at lower stimulation frequencies and that as the frequency increased, the ATP contribution decreased and NA increased (Kennedy *et al* 1986). Similar findings were made in rat superior cervical ganglia where a greater proportion of ATP was released at lower frequencies compared to Ach (Vizi *et al* 1997). The same may be true in bladders where atropine suppresses high frequency responses by 25%, but at low frequencies atropine has little effect whereas ABMA has a maximum action (Brading and Williams, 1990).

The release of both co-transmitters can be auto-regulated by both transmitters and this pre-junctional control can be dependent upon stimulation frequency and relative amounts of the co-transmitters (Sneddon *et al*, 1996). There are numerous prejunctional receptors for transmitters such as Ach, NA and also for ATP and its breakdown products, particularly adenosine. However the particular receptors, and the mechanism whereby they regulate co-transmitter release is still being investigated in different tissues. When ATP and NA are co-released in the guinea-pig vas deferens activation of β adrenoceptors located presynaptically caused an enhancement of NA release but a reduction of ATP release (Gonçalves *et al*, 1996). However at low stimulation frequencies NA release has been shown to be inhibited by NA acting on prejunctional α_2 receptors in the same tissue but this auto-inhibition is overridden at higher frequencies (Todorov *et al*, 1999). Adenosine and an A1 selective receptor agonist, 2-chloro-N6-cyclopentyl-adenosine (CPA) reduced the overflow of stimulated ATP release in the guinea-pig vas deferens (Driessen *et al*, 1994). Adenosine has also been shown to act on P1 receptors to reduce

the strength of nerve-mediated contractions in both guinea-pig and human detrusor (Ikeda *et al*, 2003) and may therefore also reduce Ach release. As ATP is rapidly degraded to adenosine following release from the nerve terminal the role of ATP in some tissues may be an inhibitory one.

Co-transmission has been described as a way of controlling the content of a neurochemical message by varying the proportions of participating neurotransmitters (Mayer and Baldi 1994). Why ATP is co-released in human detrusor but does not activate contraction directly may be due to evolutionary changes in that humans no longer need to mark territory and for social reasons need to control voiding at particular times. ATP release may not have been eliminated but may now play a more pre-junctional modulatory role in co-transmission. Any changes in the modulation due to altered storage or proportional release, or receptor presynaptic expression, as discussed above, could alter transmitter release and therefore contraction, specifically atropine-resistant contraction.

1.6.2 Purinergic receptors and the bladder

Purinergic receptors were first divided into two major classes: P1 receptors which are selective for adenosine, and P2 receptors which are selective for nucleotides. Distinguishing and characterising different classes and subtypes of the purinergic receptors has however been inhibited by the lack of selective agonists and antagonists.

There are four distinct subtypes of P1 receptors that have been characterised so far, A1, A2_A, A2_B and A3. They have seven transmembrane domains and are G-protein coupled to modulate adenylate cyclase activity and therefore the intracellular levels of cAMP (Windsheif, 1996). A1 and A3 receptors inhibit adenylate cyclase activity whilst A2_A and A2_B activate its activity (Ralevic & Burnstock, 1998). In the bladder, all the P1-subtypes are expressed, with the A2_B subtype more highly expressed than the others (Dixon *et al.*, 1996). All four subtypes are expressed in bladder urothelium with A1 localised to the apical membrane and the others are located on the basolateral membrane of umbrella cells as well as on plasma membranes of the underlying cell layers (Yu *et al.*, 2006). Adenosine has been shown to modulate the contractile properties of detrusor smooth muscle in rats, guinea-pigs and humans by reducing the force of contractions elicited by agonist and field-stimulation when pre-treated with adenosine (King *et al.*, 1997, Ikeda *et al.*, 2003). Adenosine-mediated membrane hyperpolarization and subsequent relaxation has been demonstrated, in the guinea-pig bladder, to involve a A_{2A} receptor linked to K_{ATP} channel activation via increased adenylate cyclase activity (Gopalakrishnan *et al.*, 2002). The reduction of contraction may also be due to activation of A1 receptors present on motor nerves which may inhibit further neurotransmitter release (Fry *et al.*, 2004). It has been proposed that a further role of P1 receptors in the urothelium is to modulate exocytosis in umbrella cells (Yu *et al.*, 2006).

The second major class of purinergic receptor, P2, was divided into P_{2X} and P_{2Y} based initially on pharmacological evidence in 1985 (Burnstock and Kennedy, 1985). Following this numerous other P2 receptors were demonstrated and named including P_{2z}, P_{2t} and P_{2u}. Due to the increasing number of P2 receptors and their random naming; the nomenclature was rationalised by dividing them it into two major groups termed P2X and P2Y (Abbracchio and Burnstock, 1994, Fredholm *et al*, 1994). P2X describes those receptors that are ligand-gated non-specific cation channels and P2Y describes those that are G-protein coupled. Unlike P2Y receptors, most P2X receptors are desensitised by α , β -methyleneATP.

Currently seven P2X receptors subtypes have been cloned and characterised, they are termed P2X₁ to P2X₇. Each subunit has two transmembrane domains separated by an extracellular domain of approximately 280 amino acids (North, 2006) which contains the putative ATP binding site (Gever *et al*, 2006). Channels can form as multimers of several subunits, evidence suggests that they form homomeric and heteromeric trimers (Stoop *et al*, 1999) and possibly in a “head to tail” arrangement (Jiang *et al*, 2003). The heteromeric trimers that have been identified and characterised are P2X_{2/3}, P2X_{1/5}, P2X_{2/6} and P2X_{4/6}. The characteristics of the receptor depend on the subtype combination.

RT-PCR studies have shown that in human detrusor smooth muscle tissue the subtypes of P2X receptor are present in the order P2X₁ >> P2X₄ > P2X₇ >> P2X₅ >> P2X₂ >> P2X₃ = P2X₆ = 0 (O'Reilly *et al*, 2001a). However this gives no indication of the relative importance of each receptor, just the expression magnitude and does not account for the

relative content of muscle and nerve. In cat detrusor smooth muscle P2X₄, P2X₅ and P2X₆ were not detected (Birder *et al*, 2004). The most abundant P2 receptor in human detrusor, the P2X₁, has been cloned and localised to chromosome 17 (O'Reilly *et al*, 2001a, Longhurst *et al*, 1996). The role of the P2X₁ channel in detrusor is to mediate the purinergic component of smooth muscle contraction. Nerve-mediated contraction of the bladder in mice lacking the P2X₁ receptor was reduced by ~50-70% (Vial & Evans 2000).

Activation of P2X receptors by ATP generates an inward, depolarising current in detrusor muscle carried by Ca²⁺ and Na⁺ (Kennedy, 2000). Depolarisation is sufficient to activate L-type Ca²⁺ channels to generate an action potential and Ca²⁺ influx to initiate contraction (Inoue and Brading, 1991). The influx of Ca²⁺ may lead to further increases of [Ca²⁺]_i due to Ca²⁺ induced Ca²⁺ release from intracellular stores.

There are also several subtypes of the P2Y receptor category. P2Y receptors are seven transmembrane domain G-protein coupled receptors with binding sites being identified on the 6th and 7th domains (Burnstock, 1997). There are currently eight mammalian P2Y receptors, P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃ and P2Y₁₄. There are also three non-mammalian, p2y₃, p2y₈, and p2y and also a number of orphan receptors that remain to be fully characterised. Activation of a P2Y receptor is coupled to a variety of second messenger systems. For example, P2Y₁, P2Y₂, P2Y₄, P2Y₆ are coupled to phospholipid turnover and intracellular Ca²⁺ mobilization via IP₃ generation. P2Y₁₁ activates

phospholipase C and adenylate cyclase and P2Y₁₂ inhibits adenylate cyclase activity (Sak and Web, 2002).

P2Y receptors were not identified in cat detrusor (Birder *et al*, 2004) but similar studies have not been performed on human tissue. P2Y receptors, although not a specific subtype, have been implemented in relaxation of smooth muscle in mouse and rat bladder (Boland *et al*, 1993; Bolego *et al*, 1995). Studies in the marmoset detrusor suggest P2Y mediated relaxation that may occur through activation of cAMP-dependent PKA activity (McMurray *et al*, 1998).

One study looking at the expression of P2 receptors in urothelium has shown that all P2X receptors are present in cat bladder urothelium in the basal and apical layers, although staining was more intense for P2X₃, and P2Y₁, P2Y₂, and P2Y₄ (Birder *et al*, 2004). The P2X₃ receptor has been detected in human urothelium (Sun and Chai, 2004). The study suggested ATP acted via urothelial P2X₃ receptors to transmit pain signals to the CNS and showed that the receptor was upregulated upon stretch of the urothelium. The role of all the P2 receptors in urothelium is unknown however it is likely that they play a part in the sensory functions of the urothelium. Some changes have been observed in bladder urothelium P2 receptors from humans and cats with interstitial cystitis (IC). Birder *et al* (2004) have shown that there was a significant reduction of P2X₁ and P2Y₂ receptors in cats with IC compared to normal. In human patients with IC increases in P2X₂ and P2X₃ have been observed compared to samples from control patients (Tempest *et al*, 2004, Sun *et al*, 2004). Studies with P2X₃ receptor knockout mice have shown enlarged bladder

capacity and reduced frequency of micturition (Cockayne *et al*, 2000) again consistent with a role for these receptors in transducing the sensation of bladder fullness.

The presence of P2 receptors on nerves within the bladder has also been investigated in humans and cats. Studies (Moore *et al*, 2001, Ray *et al*, 2003) have looked at the relative abundance of the P2X receptors that were co-localised with synaptic vesicles on parasympathetic nerves. They showed that all subtypes could be identified, although P2X₄, P2X₆ and P2X₇ were less abundant, present in only 6 - 18% of varicosities. Changes in the presence of P2X receptors in the sensory nerves were observed in patients with urge incontinence and sensory urgency. Urge incontinence was associated with a complete absence of P2X₃ and P2X₅ receptors (Moore *et al*, 2001) whilst in patients with sensory urgency all P2X receptor subtypes were reduced to 0.5 - 5% of that in control patients (Ray *et al*, 2003). It is likely that the loss of P2X receptors may impair both detrusor distension and contraction.

There is also evidence to suggest there are two further groups of purinergic receptors, P3 and P4. The P3-like receptor has been demonstrated through radio-labelling studies and has an affinity for adenine nucleosides and nucleotides (Yoshioka *et al*, 2001). The P4 receptor has affinity only for diadenosine polyphosphates (Pintor *et al*, 2000). Their physiological functions remain unclear at present.

1.6.3 ATP, contractile activation and bladder pathologies

Once ATP is released into the neuromuscular junction it activates purinergic receptors located on the detrusor smooth muscle as described previously. P2X₁ receptors are the predominant subtype within the bladder and the primary receptor through which atropine-resistant contractions are mediated. Any change in purinergic receptor levels particularly the P2X₁ receptor may affect the proportion of the purinergic component of contraction especially in overactive bladders. Changes to the expression of purinergic receptors throughout pathological bladders have been shown although they vary according to location (in the bladder) and the disorder. With detrusor from patients with symptomatic outlet obstruction the amount of P2X₁ receptor per smooth muscle cell was shown to be higher compared to control bladders (O'Reilly *et al*, 2001b) which would aid purinergic contraction. In contrast, in female idiopathic overactive bladders the P2X₁ receptors were significantly reduced although P2X₂ was elevated and the authors suggested a P2X_{1/2} receptor may be involved (O'Reilly *et al*, 2001a).

Changes to presynaptic receptor expression may also play a role in atropine-resistance as they may modulate the release of ATP and/or Ach. Presynaptic changes that have been observed include a reduction in P2X₃ and P2X₅ in human patients with idiopathic overactivity (Moore *et al*, 2001) and a significant loss of all P2X receptors in patients with sensory urgency (Ray *et al*, 2003). The receptor expression evidence for emergence of atropine-resistant contraction is however not conclusive and the significance of the findings remains unclear.

Another explanation for the emergence of atropine-resistant contractions observed in pathological bladder is that there is a relative change to the sensitivity of the purinergic receptors to ATP and cholinergic receptors to Ach compared to normal bladders. This possibility was investigated by measuring ATP and carbachol induced intracellular Ca^{2+} transients in isolated detrusor smooth muscle cells from stable and overactive human bladders. The ATP and Ach half-maximal concentrations (EC_{50}) and the magnitude of the Ca^{2+} transients were equivalent in the detrusor from the stable and overactive bladders (Wu *et al*, 1999). Results from this study suggested that atropine-resistance in overactive bladders cannot be attributed to changes in the sensitivity of the receptors, even though it has been shown by others that there are changes in P2X receptor expression (O'Reilly *et al*, 2001a,b).

1.6.4 ATP breakdown

Both Ach and ATP released from the nerve terminal are rapidly inactivated via enzymatic breakdown by acetylcholinesterases and ectonucleotidases respectively. The breakdown products can also act pre- or postsynaptically to alter contraction and transmitter release before they are taken back up into nerve for re-synthesis.

Ectonucleotidases sequentially degrade ATP to adenosine 5'-diphosphate (ADP), adenosine monophosphate (AMP), adenosine and inosine. Breakdown to ADP is the most important step in inactivation as ADP is less active at P2 receptors (Westfall *et al*,

1997). The enzymes are located on cell membrane surfaces and there is evidence in sympathetic nerves that a soluble enzyme is also released upon stimulation (Todorov *et al*, 1997). Ectonucleotidase activity is dependent on Mg^{2+} or Ca^{2+} ions and is optimal at pH 7.5 (Kennedy *et al* 1996) therefore any changes to the neuromuscular junction environment will effect ATP breakdown.

These enzymes are insensitive to inhibitors of other types of ATPases including ouabain, oligomycin, sodium azide and p-nitrophenyl phosphate. A number of substances have been shown to inhibit the action of ectonucleotidases including suramin and PPADS, however their action is not specific and may not be suitable when investigating the effects the enzymes have on ATP mediated contractions. A more specific ectonucleotidase inhibitor has been developed, ARL 67156 (6-N,N-diethyl- β,γ -dibromomethylene-D-ATP), it is an ATP analogue and its structure is illustrated in Figure 1.4. The effect of ARL 67156 was first demonstrated in rabbit tracheal epithelium where secretions mediated by UTP but not ATP- γ -S were potentiated and in human blood cells where it inhibited $[\gamma^{32}P]$ -ATP dephosphorylation in a concentration-dependent manner (Crack *et al*, 1995). In the guinea-pig bladder exogenous ATP-mediated, but not ABMA-mediated, contractions are increased in the presence of ARL 67156 (Westfall *et al*, 1997). Peak nerve-stimulated contractions in bladder were increased as were those in the presence of atropine (Westfall *et al*, 1997) showing that ARL 67156 enhanced the purinergic component of detrusor contraction by inhibiting ectonucleotidase activity. Ectonucleotidase activity has been shown to be reduced in human detrusor from some pathological bladders (Harvey *et al*, 2002). Human detrusor from bladders of certain pathologies (in particular obstructed bladders), and unlike stable bladders, generate

atropine-resistant contractions which may result from reduced enzyme activity. Reduced ATP breakdown may allow ATP greater access to the P2X receptors on the detrusor smooth muscle and produce atropine-resistant contractions.

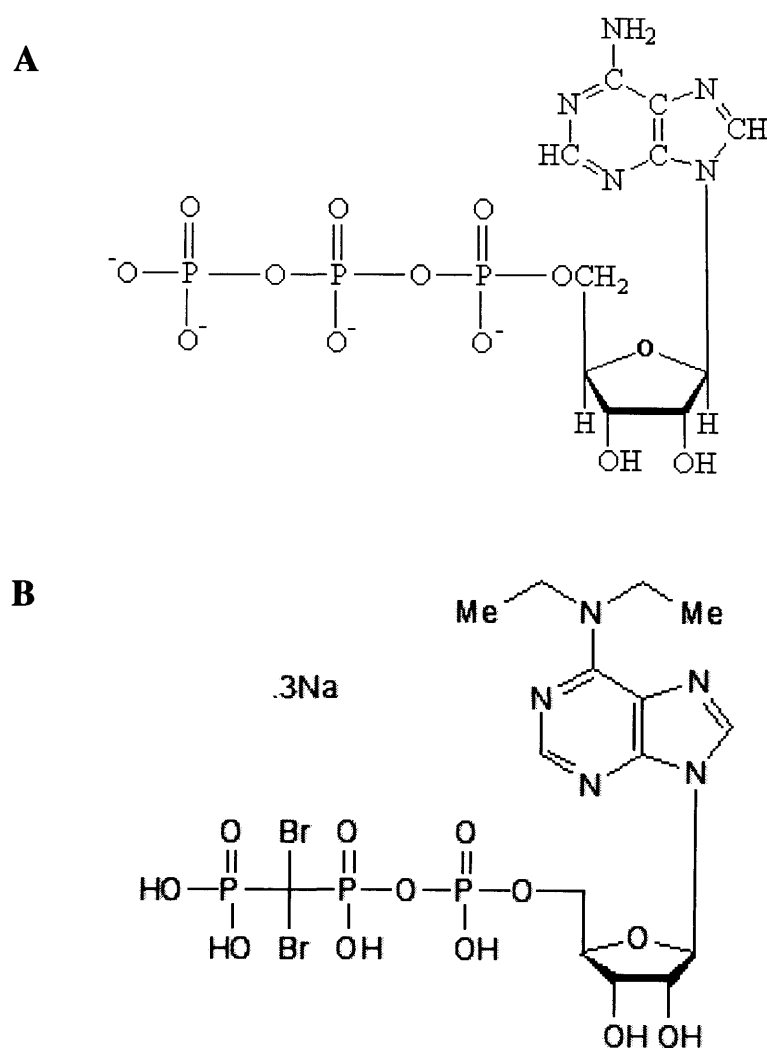
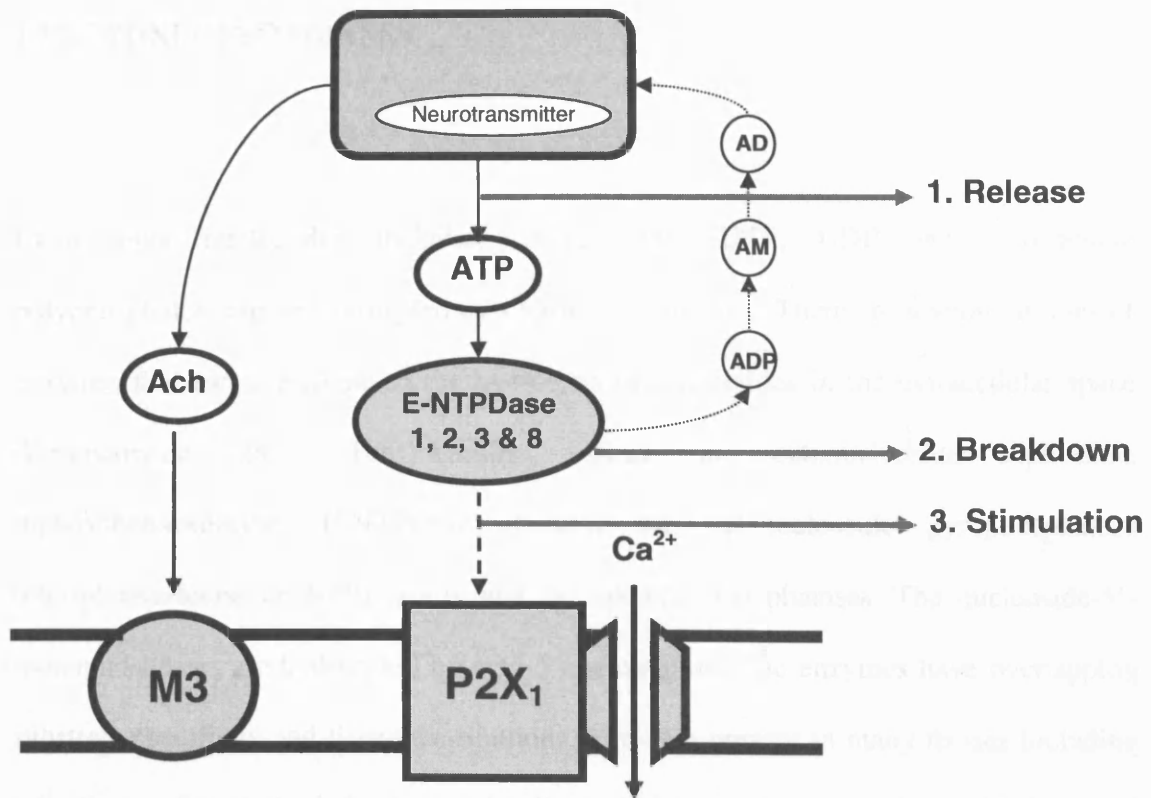


Figure 1.4 Structure of A) ATP and B) the ecto-nucleotidase inhibitor ARL 67156.

In conclusion, ATP has been established as a co-transmitter at numerous nerve-smooth muscle junctions. The presence of atropine-resistant contractions in animal and human detrusor may be attributed to activation via nerve-mediated ATP release and they may play a role in pathological bladder overactivity. The reason for the emergence of atropine-resistant contractions in human pathology may be a result of reduced extracellular breakdown, altered release or altered receptor sensitivity and it is a major objective of this thesis to gain insight into the cause of these contractions. The remaining sections of the Introduction will describe in more detail the routes whereby ATP hydrolysis is brought about and the methods available to measure extracellular ATP.



Scheme 1.1 Schematic indicating possible reasons for the emergence of atropine resistant contraction in pathological human bladder overactivity. 1) altered release of ATP, 2) reduced extracellular ATP breakdown by ectonucleotidases, 3) increased sensitivity of the smooth muscle receptors to ATP.

1.7 ECTONUCLEOTIDASES

Extracellular nucleotides including ATP, ADP, UTP, UDP and diadenosine polyphosphates, can be hydrolysed by a variety of enzymes. There are several families of enzymes that can contribute to the hydrolysis of nucleotides in the extracellular space (Zimmermann 2001). The families include the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family, the ectonucleoside pyrophosphatase /phosphodiesterase (E-NPP) family and the alkaline phosphatases. The nucleoside-5'-monophosphates are hydrolysed by ecto-5'nucleotidase. The enzymes have overlapping substrate specificity and tissue distributions. They are present in many tissues including smooth, cardiac and skeletal muscle, liver, kidney, pancreas, urinary bladder and neurones (Kennedy *et al* 1996, Plesner, 1995 and Hourani and Chown, 1989). The enzymes are located on membrane of cells or in a soluble form in fluids. It is the E-NTPDase family that is inhibited by ARL 67156 and considered to play a major role in the breakdown of ATP to AMP in the neuromuscular junction.

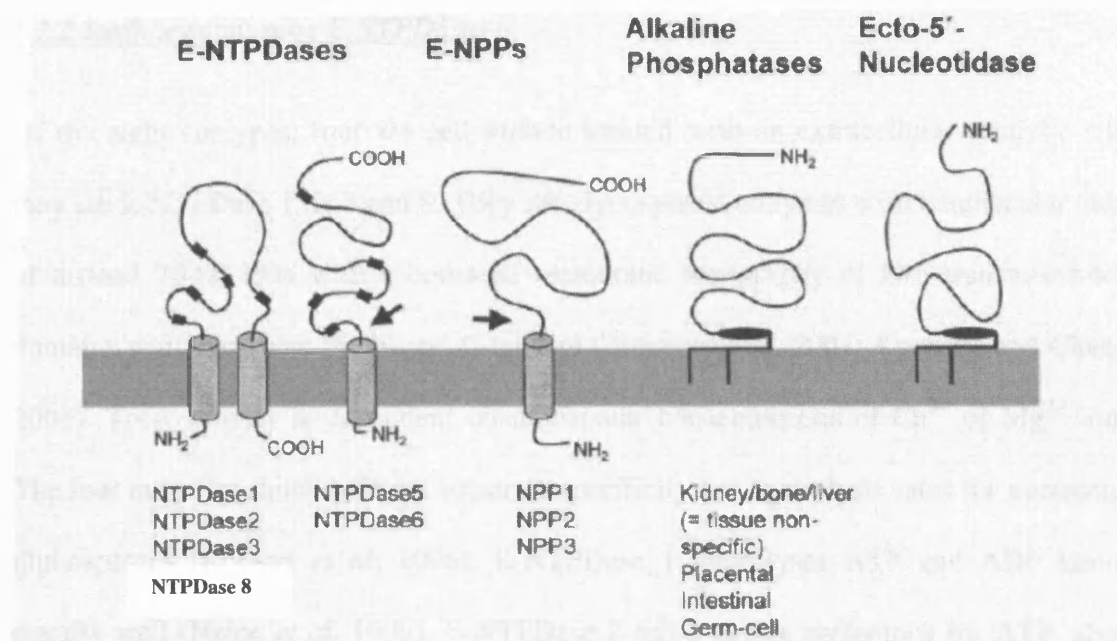


Figure 1.5 Predicted membrane topography of ectonucleotidases, E-NTPDases 1, 2, 3, 5, 6 and 8, E-NPPs 1-3, alkaline phosphatases and ectonucleotidase-5'-nucleotidase. The dark blocks on the E-NTPDases represent apyrase conserved regions that are involved in catalytic activity (modified from Zimmermann, 2001).

1.7.1 The E-NTPDase family

The E-NTPDase family are responsible for the sequential removal of the γ - and β -phosphates of extracellular tri- and diphosphonucleotides (Gendron *et al*, 2002). Currently eight members of the family have been identified, cloned and characterised, E-NTPDase 1-8. All the E-NTPDase enzymes have five highly conserved sequence domains termed apyrase conserved regions (APCR) that are involved in catalytic activity (Robson *et al*, 2006).

1.7.2 Surface membrane E-NTPDases

Of the eight subtypes, four are cell-surface located with an extracellular catalytic site; they are E-NTPDase 1, 2, 3 and 8. They are glycosylated enzymes with a molecular mass of around 70-80 kDa with a common membrane topography of two transmembrane domains which are near the N-and C-termini (Zimmermann, 2001; Knowles and Chang, 2003). Their activity is dependent on millimolar concentrations of Ca^{2+} or Mg^{2+} ions. The four enzymes show different substrate specificity and hydrolysis rates for nucleoside diphosphates (Robson *et al*, 2006). E-NTPDase 1 hydrolyses ATP and ADP almost equally well (Heine *et al*, 1999), E-NTPDase 2 has a strong preference for ATP, about 30-fold over ADP, whilst E-NTPDase 3 and 8 have a slight preference for ATP over ADP as a substrate (Zimmermann, 2001). ATP is rapidly hydrolysed to AMP by E-NTPDase 1 with minimal amounts of free ADP production preventing activation of P2Y receptors. However hydrolysis by E-NTPDase 2 results in ADP production which is slowly broken down to AMP creating agonists to P2Y receptors. Again E-NTPDase 3 and 8 activity results in an intermediate product formation of ADP and then AMP (Vorhoff *et al*, 2005; Bigonnesse *et al*, 2004). E-NTPDases can form homo-oligomeric complexes which can increase their catalytic activity, E-NTPDase 1, 2 and 3 have been found as dimers or tetramers (Wang *et al*, 1998).

1.7.3 Intracellular membrane E-NTPDases

E-NTPDase 4 has a similar general structure to E-NTPDase 1 to 3 and 8 but differs by alternative splicing (Biederbick et al, 2000). It is located entirely intracellularly, facing the lumen of cytoplasmic organelles, such as the Golgi apparatus (Wang and Guidotti, 1998) and lysosomal/autophagic vacuoles (Biederbick et al, 1999). It hydrolyses nucleoside 5'-di- and triphosphates.

E-NTPDase 5 and 6 have a predicted N-terminal hydrophobic sequence but do not have the C-terminal transmembrane domain and are localised intracellularly. E-NTPDase 5 has been located to the endoplasmic reticulum and E-NTPDase 6 to Golgi apparatus but both have also been identified as a secreted soluble form (Trombetta and Helenius, 1999; Braun et al, 2000). They have a strong preference for nucleoside diphosphates with E-NTPDase 5 hydrolysing UDP and GDP and E-NTPDase 6 GDP and IDP. It has been suggested that E-NTPDase 6 supports glycosylation reactions in the Golgi apparatus and then nucleotide hydrolysis following its release (Braun et al, 2000).

More recently discovered, E-NTPDase 7 is also located intracellularly, facing the lumen of organelles. This enzyme hydrolyses a number of nucleoside triphosphates including UTP, GTP and CTP but not ATP (Shi et al, 2001).

As mentioned above ectonucleotidase activity has been implicated in the emergence of atropine-resistant contractions in some bladder pathologies. There is evidence to suggest they play a role in other pathologies such as the proliferation of cancer cells. They have however also been shown to play positive roles in other pathologies, especially those of the cardiovascular system particularly inflammatory responses (Gendron et al, 2002).

1.7.4 Soluble ectonucleotidases

Ectonucleotidases are normally located on the extracellular membrane surface of cells however some soluble forms have also been described. The soluble enzymes could be released from nerve vesicles or could arise from proteolytic cleavage of membrane-bound enzymes. Studies have not been carried out to determine if these enzymes are released from parasympathetic nerves of the detrusor and contribute to the breakdown of ATP in this neuromuscular junction. Soluble ectonucleotidases are released upon nerve stimulation of guinea-pig vas deferens. Superfusate from only stimulated tissue was able to degrade exogenous ATP, but the effect was abolished when the tissue was stimulated in the presence of TTX implying the enzymes were released from the nerves (Todorov *et al*, 1997). There are two released soluble enzymes which hydrolyse ATP: one to ADP and AMP; and the second to AMP and adenosine (Mihaykova-Todorova *et al*, 2002). The first shows some similarities to ENTPDases and is ARL 67156-sensitive, whilst the second has similarities to ectonucleotidase-5'-nucleotidase (Westfall *et al*, 2002).

1.8 MEASUREMENT OF ATP

ATP is a ubiquitous substance with numerous functions within biological systems. Recently its role as an important transmitter has been begun to be established in both the central and peripheral nervous system, playing a role not only in the bladder, as discussed above, but in all systems and tissues. ATP has been identified as a neurotransmitter through electrophysiological, pharmacological and direct measurement studies. Silinsky and Redman (1996) used patch electrodes to measure activation of ATP-gated ion channels via stimulation of frog cutaneous pectoris nerve muscle preparations. The experiments suggested that ATP was synchronously released with Ach in response to nerve impulses. The use of agents such as atropine and ABMA has highlighted the role of ATP in detrusor contraction of the bladder of most mammalian species.

1.8.1 ATP measurement techniques

Various techniques have been employed to measure directly ATP release from tissues and nerves including the use of radio-labelled ATP, fluorescence detection HPLC and the luciferin–luciferase assay. Each method has associated advantages and drawbacks. A consideration when using radiolabelled purines is that they may not follow the uptake and release of endogenous ATP and it is difficult to distinguish ATP from other purines released by the tissue (Sperlagh and Vizi, 1996). The luciferin–luciferase assay is simple

and has a high sensitivity, in the femtomolar range, whilst the use of HPLC enables ATP breakdown products to be also measured from the same sample. Superfusate samples taken at 10 second intervals during 60 second stimulation of guinea-pig vas deferens were analysed by HPLC-fluorescence detection to show co-transmitter ATP release with NA was likely to be from separate exocytotic vesicles (Todorov *et al*, 1996). The study used 2-chloroacetaldehyde to produce the fluorescent derivatives ethano (ϵ)-ATP to enable the ATP and its breakdown products to be measured. The luciferin-luciferase assay was used to measure ATP release from human detrusor following varying degrees of stretch and electrical stimulation (Kumar *et al*, 2004). A sample of the solution surrounding the tissue in the organ bath was taken following intervention and measured and compared to a basal recording. Santos *et al* (2003), used the luciferin-luciferase assay to measure ATP release from the rat phrenic nerve: they showed that the release was frequency dependent over 1-5 Hz. As before the ATP measurement was made by taking samples of the bath solutions and comparing them to basal levels.

Although the techniques above provide useful and sensitive methods for measuring direct ATP release they are difficult to use in vivo and many rely on the measurement of ATP in samples taken from the solution surrounding the preparation. Superfusate sample collection means that there is a delay between ATP release and ATP measurement and this can lead to degradation of ATP and inaccurate measurements. ATP will also be broken down by any ATPases collected in the sample. Another major drawback of these techniques is that they are unable to measure ATP release in real-time during physiological activity. The recent development of microelectrode biosensors provides a

novel way of measuring endogenous ATP release in real-time, allowing for the timing, dynamics and quantity of ATP release to be recorded (Llaudet et al, 2004; Dale et al, 2005).

1.8.2 Microelectrode biosensors - characteristics

Biosensors are described as any sensor that uses a biological component to bind specifically to an analyte of interest and provide a physical signal that is a function of the amount of analyte (Dale et al, 2005). The initial microelectrode biosensors for purines were developed by Dale (1998) for the measurement of adenosine in frog embryos. The benefit of microelectrodes is that they give good temporal and spatial resolution and are minimally invasive, especially when compared to other methods. The adenosine and ATP electrodes are prepared in a similar way and work on the same principles. Enzymes are entrapped in a robust, highly porous biolayer which is thinly coated around a platinum microelectrode (Figure 1.6). The enzymes are entrapped so that they are not lost from the electrode and therefore it can be used repeatedly. With the ATP electrode the enzymes present are glycerol kinase and glycerol-3-phosphate oxidase. In the first step of the enzyme cascade glycerol kinase catalyses the transfer of a phosphate group from ATP to glycerol. Glycerol must therefore be present throughout the experiment for the electrode to be active. In the second step hydrogen peroxide is produced by the oxidation of glycerol-3-phosphate. The H_2O_2 thus generated can be amperometrically detected when oxidised by the Pt electrode polarised to 500-700 mV relative to an Ag/AgCl reference.

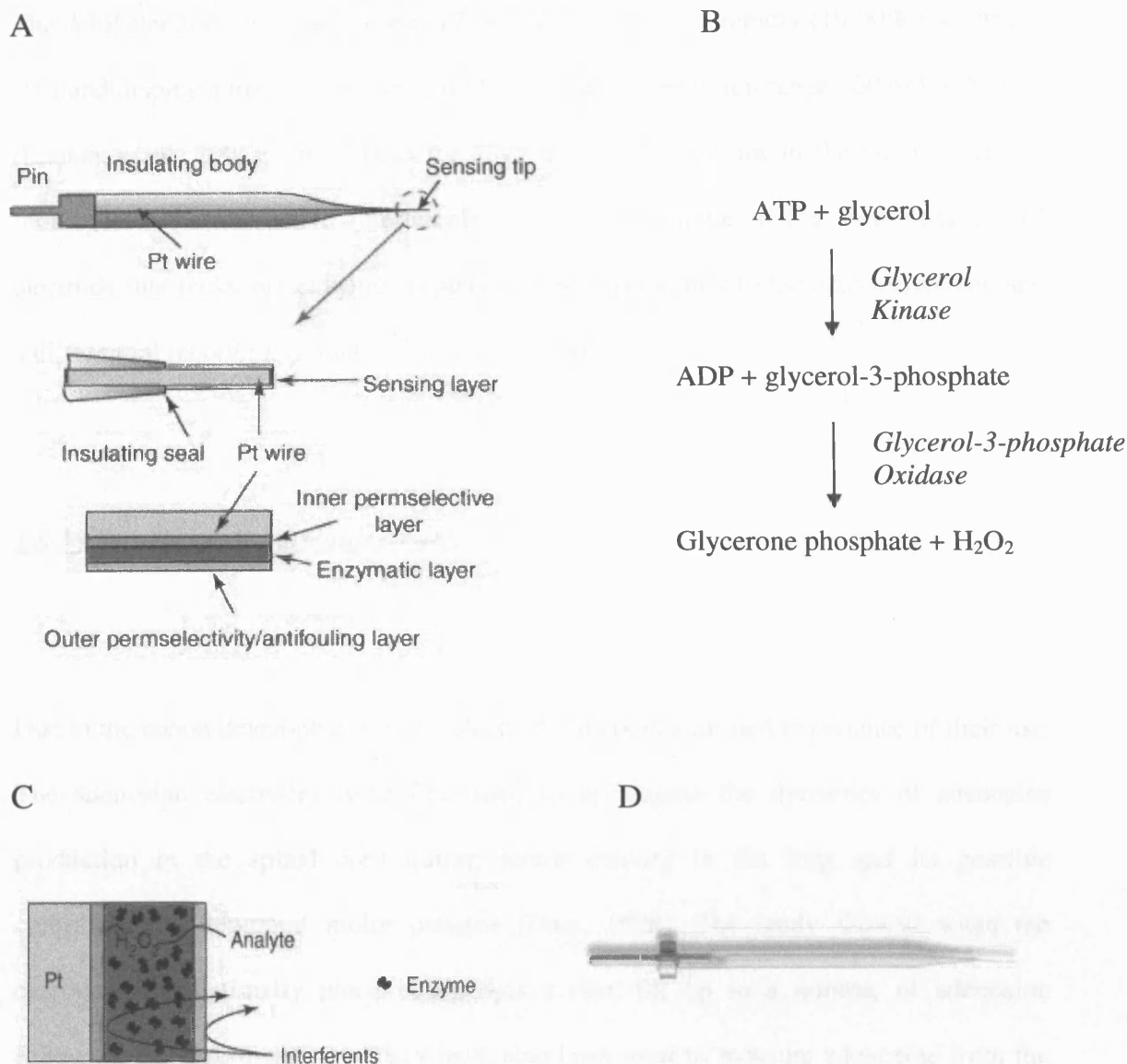


Figure 1.6 Principle of the ATP microelectrode biosensor. A) Drawing of the ATP electrode structure showing the overall electrode and the layers of coatings surrounding the Pt wire which will detect ATP. B) The enzyme cascade used to measure ATP. C) The analyte, ATP, diffuses into the enzyme layer and is converted to H_2O_2 which diffuses to the electrode. D) Image of the ATP biosensor used to measure real time release of ATP.

The ATP electrodes have a diameter of 50-100 μm , respond rapidly (10-90% rise time < 10s) and display a linear response to ATP over the concentration range 200 nM to 50 μM (Llaudet et al, 2005). To account for any electroactive species in the environment or 'non-specific' electroactive interferents that may contribute to the recordings a null electrode that lacks any enzymes is placed in close proximity to the active electrode and a differential recording is made (Gourine et al, 2005).

1.8.3 Microelectrode biosensors – uses

Due to the recent development of the electrodes there is a limited experience of their use. The adenosine electrodes were first used to investigate the dynamics of adenosine production in the spinal cord during motor activity in the frog and its possible contribution to temporal motor patterns (Dale, 1998). The study showed when the electrode was optimally placed there was a rise, for up to a minute, of adenosine following the end of activity. They have also been used to measure adenosine from the dorsal surface of the brainstem and from with the nucleus tractus solitarii during the defence response caused by hypothalamic stimulation in the rat (Dale et al, 2002) and to study the role of adenosine release during hypoxia in rat hippocampal slices (Freguelli et al, 2003).

The use of the ATP electrode so far includes measuring the release of ATP from the spinal cord of *Xenopus* embryos during swimming (Llaudett et al, 2004, 2005). The electrodes were placed in alignment with the ventral part of the spinal cord and transient ATP release was observed during the swimming episode. The role of ATP in control of respiratory activity has been investigated with ATP electrodes (Gourine et al, 2005). The group was able to show that hypoxia induced a marked increase in ATP and that the ATP is produced throughout the ventrolateral medulla in locations corresponding to the ventral respiratory column. In the most recent study to utilise the ATP electrode, Pearson et al (2005), showed that the retinal pigment epithelium (RPE) regulates proliferation in the neural retina via the release of ATP.

The experiments with adenosine and ATP electrodes have shown they have a wide application both in vivo and in vitro. In this thesis the ATP microelectrode biosensor will be used to try to measure ATP in real time under sensitive stimulation parameters of contraction of the detrusor smooth muscle.

1.9 AIMS AND OBJECTIVES OF THESIS

Hypothesis:

Nerve-mediated ATP release plays a variable role in contractile activation in detrusor smooth muscle from animal and human bladders. This variable role is due to differential breakdown or release of extracellular ATP.

Aims of the thesis:

The aim of this project was to investigate the role of ATP signalling in the neuromuscular junction of guinea pig and human detrusor smooth muscle with particular emphasis on ATP breakdown by ectonucleotidases. The effects of ectonucleotidase modulators were investigated on the contractile activity of electrically field stimulated isolated detrusor preparations. Ectonucleotidase activity was determined by measuring ATP degradation in detrusor samples using a luciferin-luciferase assay. Real-time PCR was used to establish relative levels of ectonucleotidase enzymes in samples of human detrusor. Nerve mediated release of ATP in guinea pig detrusor strips was measured in real time using microelectrode biosensors.

Objectives:

The following experimental objectives were addressed through the experiments performed in this study, to determine the role of ATP release and breakdown in detrusor.

- i.) To determine the effect of altering ectonucleotidase activity on nerve-induced contractions in guinea-pig and human detrusor, and whether this differs between tissue from stable and overactive human bladders.
- ii.) To determine the rate of ectonucleotidase activity in guinea-pig and human detrusor and whether this differs between detrusor samples from stable and overactive human bladders.
- iii.) To determine if there are any differences in the expression of ectonucleotidase 1, 2, 3 or 5 in detrusor samples from stable and overactive human bladders.
- iv.) To establish if novel ATP sensitive microelectrode biosensors are suitable to measure ATP release in real time during nerve-stimulated contractions of guinea-pig detrusor.

2.0 Materials and Methods

2.1 SOLUTIONS AND CHEMICALS

2.1.1 Physiological Solutions

The physiological saline solution used for experiments was a modified Tyrode's solution buffered with either N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) or $\text{NaHCO}_3/\text{CO}_2$. The compositions of these solutions are shown in Tables 2.1 and 2.2. Fresh Tyrode's was prepared daily using deionised water from a Purite reverse osmosis water filter (Purite Ltd, Thames, Oxon, UK). Stock solutions of 1 M KCl, MgCl_2 , and NaH_2PO_4 were prepared in deionised water and CaCl_2 was purchased as a 1 M solution (BDH). All stock solutions were stored at 4°C. Glucose, Na pyruvate, NaCl, NaHCO_3 and HEPES were added as solids. Solids were weighed on a Sartorius balance (Sartorius Ltd, Epsom, Surry) and liquids were measured using a measuring cylinder or variable volumetric pipettes.

$\text{NaHCO}_3/\text{CO}_2$ -buffered solutions, used for experiments utilising ATP biosensors, were gassed with 95% O_2 , 5% CO_2 mixture to maintain a pH of 7.33 ± 0.02 at 37°C (see section 2.3). An aliquot of a 5 M glycerol stock was added to this modified Tyrode's solution to give a final concentration of 2 mM. Glycerol was added as it was a co-substrate required for the operation of the ATP biosensor (see section 2.8).

The Tyrode's solution used in the tissue strip experiments was buffered with HEPES. The pH of the solution was adjusted to 7.3 using 1 M NaOH and a pH meter (Meter - Solex digital pH/MV meter, Taiwan R.O.C. Electrode – BDH glass+). The solution temperature was maintained at 37°C during experiments.

Modifications were made to the HEPES-buffered Tyrode's solution for use in the ATP degradation experiments measuring ecto-ATPase activity in detrusor. The modification made to the HEPES buffered Tyrode's was to remove all calcium and add 5 mM ethylene diamine tetra acetic acid (EDTA). The pH of the solution was adjusted to 7.8 as required for the luciferin-luciferase assay. This solution was used ice-cold to dilute samples and to terminate ATP degradation.

An ungassed, nominally Ca^{2+} -free HEPES buffered Tyrode's solution with the pH adjusted to 7.6 was used for transport and storage of human tissue samples. The composition is shown in Table 2.3. This solution was also used for the storage of guinea pig bladders.

Table 2.1 Composition of Tyrode's solution

Chemicals	Concentration (mM)
NaCl	118.0
NaHCO ₃	24.0
KCl	4.0
MgCl ₂ .6H ₂ O	1.0
NaH ₂ PO ₄ .2H ₂ O	0.4
CaCl ₂ .6H ₂ O	1.8
Glucose	6.1
Na pyruvate	5.0

Table 2.2 Composition of HEPES Tyrode's solution

Chemicals	Concentration (mM)
NaCl	132.0
HEPES	10.0
KCl	4.0
MgCl ₂ .6H ₂ O	1.0
NaH ₂ PO ₄ .2H ₂ O	0.4
CaCl ₂ .6H ₂ O	1.8
Glucose	6.1
Na pyruvate	5.0

Table 2.3 Composition of Ca⁺² -free HEPES Tyrode's solution

Chemicals	Concentration (mM)
NaCl	105.4
NaHCO ₃	22.3
KCl	3.6
HEPES	19.5
MgCl ₂ .6H ₂ O	0.9
NaH ₂ PO ₄ .2H ₂ O	0.4
Glucose	5.4
Na pyruvate	4.5

2.1.2 Chemicals and Drugs

The chemicals and drugs used as interventions during the experiments were added to the appropriate Tyrode's solution to give the required concentration.

Atropine, 6-N,N-diethyl- β - γ -dibromoethyle-D-adenosine-5'-triphosphate trisodium (ARL), disodium-adenosine triphosphate (Na_2ATP), α - β -methylene-adenosine triphosphate (ABMA), carbachol and tetrodotoxin (TTX) were added to the Tyrode's solutions from 10 mM stocks. The stocks were prepared by dissolving the solid chemicals in deionised water and were stored at 4°C or in small aliquots at -20°C.

Nifedipine (10 mM) stock solution, prepared in dimethyl sulphoxide (DMSO), was made on a daily basis due to its instability in light.

High potassium chloride solutions, 120 mM, were prepared by addition of a 1 M stock solution to the glycerol-containing Tyrode's solution.

Apyrase solid was dissolved in HEPES-Tyrode's solution to give a stock concentration of 20 U.ml⁻¹. The concentration was calculated by the enzyme activity of each batch. Aliquots of 500 μ l were frozen at -20°C in eppendorf tubes. Once thawed, 500 μ l of HEPES Tyrode's was added to the aliquots to give a final concentration of 10 U.ml⁻¹.

Adenosine 5'-triphosphate bioluminescent assay kit, containing ATP assay mix; ATP assay mix dilution buffer; and ATP standard was purchased from Sigma. The assay mix was a lyophilized powder containing luciferase, luciferin, MgSO_4 , dichloro-diphenyl-trichloroethane (DDT), EDTA, bovine serum albumin (BSA) and tricine buffer salts. It was reconstituted using 5 ml deionised water. Any dilutions of the assay mix were made using the dilution buffer.

Chemicals were purchased from Sigma unless stated otherwise.

2.2 TISSUE COLLECTION AND PATIENT GROUPS

2.2.1 Human detrusor

Human detrusor tissue samples were obtained from open surgery with local ethical committee approval from University College London Hospital Trust and informed patient or guardian consent.

Biopsies were dissected from the dome region in a posterior to anterior direction. Samples are immediately placed and transferred from the surgical theatre in Ca^{2+} -free HEPES buffered Tyrode's solution. Samples were stored at 4°C and could be used for up to 48 hours.

Samples were divided into different groups depending on results of urodynamic tests or pathology:

- i) 'Control' group consisting of stable bladders. Control samples were obtained from patients undergoing a cystectomy for bladder cancer or removal of a localised bladder tumour. Samples dissected from whole bladders removed by cystectomy were taken from sites unaffected by the tumours.
- ii) 'Overactive' group. Patients in this group had urodynamically-proven bladder overactivity. The overactivity was associated with neurological disorders, bladder outflow obstruction or was idiopathic. These samples were

obtained, in the main, from ileocystoplasty procedures. Samples were obtained from patients with urodynamically proven bladder overactivity.

2.2.2 Guinea Pig detrusor

Male Dunkin-Hartley guinea pigs (400-600 g) were sacrificed by cervical dislocation according to United Kingdom Home Office specifications, under a personal and project licence to Professor CH Fry. The bladder was dissected out and immediately placed in Ca^{2+} -free HEPES buffered Tyrode's solution. The bladder was immediately used or was stored at 4°C for up to 48 hours before use.

2.3 ISOMETRIC TENSION MEASUREMENT

2.3.1 Muscle strip preparation

Both human detrusor samples and whole guinea pig bladders were placed in dissection dishes containing Ca^{2+} -free HEPES buffered Tyrode's solution. Samples were held in position with fine gauge syringe needles.

The guinea pig bladder was opened and the urothelium was dissected away with fine dissection scissors. The detrusor layer contained densely packed muscle bundles which ran longitudinally from the base to the dome. The bundles were a greyish-white colour and are slightly translucent. Longitudinal strips were cut avoiding the trigone.

When present, the serosa was removed and the urothelium dissected away from human samples. Human detrusor bundles appeared in random orientation and were pearly-white in colour with a slight translucent quality. Longitudinal arrangements of muscle bundles dissected for strips were identified using a binocular microscope (Nikon AL5, Nikon Corporation, Tokyo, Japan).

A segment of the human samples was frozen and stored in liquid nitrogen (N_2) for subsequent experiments.

Where possible, strips were dissected to consist of discrete bundles of muscle however this was not always possible with human samples. The strips had approximately the following dimensions: diameter 1-2mm, length 4-6mm. The strips were tied at either end with surgical suture thread (Persalls sutures, cornea silk, US7/0).

2.3.2 Tissue mounting and equipment set-up

Tissue strips were transferred to the trough of a horizontal organ bath. Warmed water to 37°C flowed in channels either side of the trough to maintain the temperature of the tissue and solution within the trough. In the trough the tissue was fastened to a static hook using suture. The other end was attached, again with suture to an isometric force displacement transducer (Model FT.03, Grass instrument Co. USA). Using micromanipulators (Prior instruments Ltd., Bishop's Stortford, Hertfordshire, UK) the position of the tissue was adjusted to ensure it did not touch either the sides or the bottom of the trough and that the tissue was completely immersed in solution.

The tissue was superfused with HEPES-buffered Tyrode's solution which was maintained at 37°C in a water bath. The superfusate flowed, under gravity, through a water-jacketed system to the trough at rate of 5 ml.min⁻¹. The volume of the organ bath was approximately 0.064 cm³.

The organ bath was attached to a heavy metal table and stage. The stage was placed in sand tubs in order to minimise mechanical interference to the recordings by external vibrations.

The force displacement transducer was connected to a bridge amplifier with variable gain (TB4M Transbridge, World Precision Instruments, US). The amplifier was then connected to a chart recorder with a low-pass filter of corner frequency 10 Hz (Multitrace 2; ORMED Ltd, Hertfordshire, UK). The tissue was stimulated by pulse trains from a constant-current source via platinum (Pt) electrodes in the walls of the trough. The pulses were generated by a stimulator (Stimulator Model 200, Palmer Bioscience, Sheerness, Kent) and gated by a programmer (Model 150, Palmer Bioscience, Sheerness, Kent). The stimulation was about 1.5 times threshold by adjusting output voltage (30-40 V). The protocol is described in section 2.3.1.

At the end of the experiment the dimensions of the muscle strip were measured to determine the cross-sectional area of the muscle in order to normalise force values (mN. mm^{-2}).

2.3.3. Calibration of tension transducer

To calibrate the tension transducer a range of weights were hung vertically from the force transducer hook. The deflections, measured in mm, were plotted as a function of the force exerted by gravity. The force F in Newtons (N) being:

$$F = m \cdot g$$

where m = mass of weight (kg), g = acceleration due to Earth's gravity (9.81 m.s^{-2})

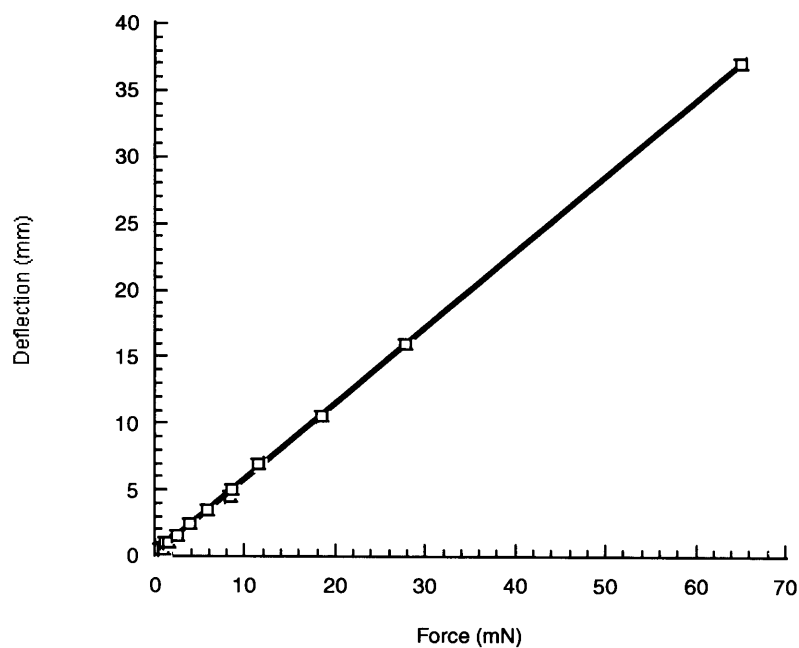


Figure 2.1 Calibration plot of tension transducer.

2.3.4 Apyrase protocol

The experimental protocols were the same for guinea pig and human detrusor tissue.

Once fastened in the organ bath, muscle strips were stretched using micromanipulators to approximately 1.5-times their relaxed length. This was to ensure maximum contraction tensions were obtained upon electrical stimulation.

Electrical field stimulation elicited TTX-sensitive phasic contractions of the tissue strip via the embedded motor nerves. The stimulation parameters were 3-second pulse trains (0.1 ms pulse width) every 90 seconds at varying frequencies.

Guinea pig and human muscle strips were stimulated at 8 Hz and 20 Hz respectively until the contraction size attained was stable. The time required to reach a stable response was between 30 minutes and 1 hour. The particular frequencies were selected to give a contraction of about half-maximal force. Force-frequency curves were generated by stimulating the strips at a range of frequencies between 1 and 40 Hz.

Due to the cost of apyrase it was not possible to allow the apyrase-containing superfusate to flow through the bath. Therefore during all apyrase interventions the superfusate flow was suspended ('no flow'). For these interventions 1 ml of 10 U.ml⁻¹ apyrase was warmed to 37°C and added to the bath via a syringe and a three-way tap attached to the tubing just before it entered the bath. As a result of apyrase interventions being performed in such a way an equivalent 'no flow' situation was performed with the tissue

in HEPES Tyrode's without addition of apyrase. In both instances flow was returned when the intervention was completed. The maximum time for the flow to be suspended was 15 minutes. The 'no flow' situation would be considered as the control to which apyrase interventions should be compared.

The effect of apyrase on the force frequency curve and fixed-frequency contractions of guinea pig and human detrusor were investigated. The effect of apyrase in the presence of atropine (1 μ M) was also studied.

2.3.5 Data analysis

Empirical fits to force-frequency $T = \frac{T_{\max} \cdot f^n}{k_{1/2}^n \cdot f^n}$ data was performed by least squares analysis. T_{\max} is the estimated maximum effect, $k_{1/2}$ is the half-maximal frequency to generate half-maximal response. f is the frequency of stimulation, and n is a constant. For $n = 1$ the relationship would describe a rectangular hyperbola, for $n > 1$ the curve would assume an increasing degree of sigmoidicity. No physiological significance was associated with the value of n . It was used only to optimise the curve fit.

2.4 TOTAL RNA ISOLATION

Before any experiment involving RNA or DNA was carried out the work-bench and all apparatus were cleaned with RNase Zap (RNase Zap, Ambion) to prevent contamination.

2.4.1 Preparation of RNeasy Fibrous Tissue Mini Kit

The kit purchased to isolate RNA from the human detrusor samples was RNeasy Fibrous Tissue Mini Kit, (QIAGEN Ltd, West Sussex). Buffers RLT, RPE, RDD and RW1, RNase free water and protein kinase-K were contained in the kit, whilst DNase-1 was purchased separately from Ambion Inc. The kit was prepared and used as described in the instruction handbook with some small alterations.

Initial Preparations

- 10 μ l of β -mercaptoethanol (Sigma) was added to every 1 ml of Buffer RLT. Buffer RLT is a lysis buffer containing the highly denaturing guanidine isothiocyanate. This inactivates RNases to ensure isolation of intact RNA.
- Buffer RPE was diluted to 1 in 4 with 96-100% ethanol to give a suitable working concentration
- DNase 1 stock solution was prepared by dissolving the DNase-1 solid in 550 μ l RNase free water. The solution was mixed but not vortexed. DNase-1 solution is used to digest any DNA present in the sample.

2.4.2 Preparation of Tissue

To isolate RNA from each human detrusor tissue sample 10-30 mg of each frozen sample was rapidly cut and weighed. The frozen tissue sample pieces were then placed in 600 μ l of Buffer RLT and homogenised using a rotor-stator homogeniser. Homogenisation continued until the sample was cloudy and uniformly homogeneous (usually 40-60 seconds). The homogenate was transferred to a 2 ml eppendorf tube.

2.4.3 RNA Isolation

580 μ l of RNase-free water was added to the homogenate followed by 20 μ l of proteinase-K solution which was mixed by pipetting. Proteinase-K solution is added to digest proteases, it contains a subtilisin-type protease isolated from the saprophytic fungus *Tritirachium Album*. The solution was incubated for 10 minutes at 55°C in a heating block (Eppendorf Thermomixer 5436) and then centrifuged for 3 minutes at 10,000 x g (Eppendorf Centrifuge 54151). The supernatant was pipetted into a new 1.5 ml micro-centrifuge tube, avoiding the pellet of tissue debris. In some samples a thin layer or film was present on top of the supernatant and efforts were made to avoid transferring any of this layer with the supernatant. 600 μ l of 100% ethanol was added and mixed to provide good binding conditions of the RNA to the column.

From this supernatant solution 700 μ l was pipetted onto an RNeasy mini spin column (contains a silica gel membrane that binds RNA) which in turn was placed in a 2 ml

collection tube. This was centrifuged for 15 seconds at 8000 x g. The remainder of the supernatant solution was pipetted onto the same column and centrifuged in the same way. The flow-through was discarded into a separate waste container. To wash the column 350 µl of Buffer RW1 was pipetted onto the column and centrifuged 15 seconds at 8000 x g; again the flow-through was discarded.

DNase-1 solution was prepared by adding 10 µl of DNase-1 stock solution to 70 µl of Buffer RDD and mixing gently (DNase-1 is very sensitive to physical denaturation). The 80 µl of DNase-1 solution was pipetted onto the membrane of the RNeasy mini column, this was then incubated at room temperature for 15 minutes. Once digestion was complete 350 µl of Buffer RW1 was pipetted onto the column. Following centrifugation (15 seconds, 8000 x g) the flow-through and collection tube were discarded. The column was transferred to a clean 2 ml collection tube and 500 µl of Buffer RPE was pipetted onto it. To wash, the tube was closed, centrifuged (15 seconds, 8000 x g) and the flow-through discarded. Another 500 µl of Buffer RPE was added to the column and centrifuged for 2 minutes at 8000 x g. In order to dry the membrane and reduce the possibility of buffer carry-over, the column was placed into a clean collection tube and centrifuged for a further 1 minute.

To elute the RNA, the column was again transferred to a new 1.5 ml collection tube and 30 µl of RNase-free water was added to the column. This was centrifuged for 1 minute (8000 x g). The elute containing the RNA was stored at -20°C.

All discarded flow-through was mixed with 100% ethanol before being disposed.

2.4.4 Determination of RNA quality

To ensure the isolated RNA was of an acceptable quality to use for further studies the samples were analysed using an RNA 6000 Nano Labchip Kit (Agilent Technologies, UK) and the Agilent 21000 Bioanalyser (Agilent Technologies, UK). The technique enables minimal samples to be analysed by “lab on a chip” technology. Samples are electrophoretically separated and detected by laser-induced fluorescence, and translated into gel-like images and electropherograms. The kit was prepared and used as described in the guide.

- Preparing the gel

The reagents were left to stand at room temperature for 30 minutes to equilibrate. An aliquot (550 µl) of RNA 6000 Nano gel matrix was pipetted into the top receptacle of a spin filter. This was centrifuged for 10 minutes at 4000 rpm (Eppendorf Centrifuge 54151) and then the spin filter was discarded. 65 µl aliquots of the filtered gel were placed into RNase-free microfuge tubes and stored at 4°C.

- Preparing the gel-dye mix

Reagents were allowed to equilibrate to room temperature for about 30 minutes making sure the dye concentrate was protected from light. The RNA 6000 Nano dye concentrate was vortexed and then spun down. 1 µl of this dye was added to a 65 µl aliquot of filtered gel, vortex mixed and then centrifuged for 10 minutes at 14000 rpm (Eppendorf Centrifuge 54151).

– Loading the gel-dye mix

A new Nano chip was opened and placed on the chip priming station of the bioanalyser. 9 μ l of the gel-dye mix was pipetted into the well labelled **G**. The chip priming station was then closed and the plunger pressed down and held for 30 seconds. The plunger was then slowly released and the priming station opened. 9 μ l of the gel-dye mix was pipetted into the two wells marked **G**.

– Loading the RNA 6000 Nano marker

5 μ l of the RNA 6000 Nano marker was pipetted into the well marked with the symbol of a ladder and into each of the twelve sample wells.

– Loading the ladder and sample

RNA 6000 ladder (Ambion Inc) was pipetted into an RNase-free microcentrifuge tube and heat denatured at 70°C for 2 minutes. 1 μ l of the ladder was pipetted into the well marked with the ladder symbol and 1 μ l of each RNA sample was pipetted into the sample wells. Wells that were not used to analyse a sample had 1 μ l of RNA Nano maker added. The chip was placed in the vortex mixer and vortexed at 2400 rpm for 1 minute.

– Setting up the bioanalyzer

Before the chip was placed in the bioanalyzer (Aligent 2100) the electrodes were cleaned using RNaseZAP and RNase-free water in the electrode-cleaning chip. The electrodes were then allowed to air dry before the sample chip was inserted and the

lid closed. The computer software, 2100 Expert, was used to select the appropriate assay (electrophoresis, RNA, Eukaryote Total RNA Nano) and start the bioanalyzer. Once the run was complete the chip was removed and discarded and the electrodes cleaned as before.

2.4.5 Determination of RNA concentration

The concentration of each human detrusor RNA sample to be used in expression studies was determined by using a Genequant RNA/DNA calculator (Genequant Pro, Amersham Biosciences). The instrument was set to measure the absorbance at 260 nm and 280 nm to determine the RNA concentration. All samples were defrosted but kept on ice whilst the measurements were taken.

Small glass capillary tubes with a volume of 7 μ l were dipped into the sample solution and held until the solution filled the tube. One end of the tube was then sealed using plasterscine. The capillary tube was then carefully placed in the Genequant and the absorbance taken and recorded. The capillary tube was then removed and discarded. Before the samples were measured a capillary filled with RNase-free water was measured in order to set a background reading.

2.5 REVERSE TRANSCRIPTION

RNA is very unstable and will degrade easily. Reverse transcription to cDNA gives an identical population that is stable at high temperatures and therefore samples are able to undergo the polymerase chain reaction (PCR). In the reaction RNA is denatured by heat and then cooled in the presence of random hexamers (short random repeats of six to ten nucleotides) which bind to complimentary sequences on RNA. Addition of a mixture of deoxyribonucleotides (dNTP) and a DNA polymerase enzyme in the presence of a suitable buffer initiates the polymerisation reaction.

2.5.1 Reagents

5 x PCR Buffer II (contains MgCl_2 as the enzyme requires Mg^{2+} to function), 10 mM GeneAmp dNTP blend, MuLV (Moloney murine leukaemia virus) Reverse Transcriptase, RNase Inhibitor and random hexamers were purchased from Applied Biosystems (USA). All reagents used were thawed on ice and then kept on ice whilst in use.

2.5.2 Protocol

A mastermix of the reagents was prepared by adding them in the following proportions:

5 x PCR Buffer II	1x (4µl per well)
10mM dNTP blend	1 mM each dNTP (2µl per well)
RNase inhibitor	1U.µl ⁻¹ (1 µl per well)
Random hexamers	2.5 µM (1 µl per well)

The mastermix was pipetted into the required wells of a 96-well plate (MicroAmp® Optical 96-well Reaction Plates, Applied Biosystems). To each well an appropriate volume of sample was added to give a concentration of between 100 ng and 1 µg of RNA. The final solution volume of each well was 20 µl, the remainder of this volume was made up with RNase/DNase free water. Each sample was prepared in triplicate as + reverse transcriptase reactions. A negative (-) reverse transcriptase reaction was also prepared and was used as a control for DNA contamination, if there was no reverse transcriptase present no cDNA could be made. On each plate four water controls were included, this is to check the reliability of the reagents. The plate was incubated for 10 minutes at room temperature to allow the extension of hexamers. Then to the + reverse transcriptase reaction wells 1 µl of MuLV reverse transcriptase was added. To the – reverse transcriptase reaction wells and the water controls 1 µl of water was added. Caps (MicroAmp® Optical Caps, Applied Biosystems) were placed on the plate and pushed down to seal. The plate was then gently mixed.

The plate was placed in a thermal cycler (Peltier Thermal Cycler, DNA Engine DYAP™ MJ Research). The reverse transcription protocol was as follows:

42°C	15 min	reverse transcribe
99°C	5 min	denature
5°C	5 min	cool

Once the reaction was complete and the cDNA made the plate was stored at -20°C for the samples to be used in further studies.

2.6 RELATIVE EXPRESSION OF TAQMAN® RT-PCR

TaqMan® enables real-time measurements of accumulating polymerase chain reaction (PCR) products using a dual-labelled TaqMan fluorogenic probe system (Medhurst et al, 2000). The technique utilises *Taq* DNA polymerase (from *Thermus aquaticus*) which is complexed with an antibody and called *Taq* Gold. *Taq* has a 5 prime to 3 prime (5'-3') DNA polymerase activity (adds nucleotides to the 3' hydroxyl group of oligonucleotide primers). It has exonuclease activity, also 5'-3', which removes nucleotide from the 5' end of any oligonucleotide that is bound to the template strand down-stream of the annealed primer. In the Taqman reaction is a third oligonucleotide, a reporter and quencher labelled probe. The probe anneals downstream (towards the 3' end) of the forward primer. The reporter and quencher are covalently attached fluorophores with the reporter dye at the 5' end and the quencher dye at the 3' end. The reporter dye fluoresces (achieved by Förster Resonance Energy Transfer, FRET) when illuminated by laser light, but is quenched (fluorescence reduced) when the quencher dye is in close proximity. When the probe anneals downstream of the primer the *Taq* exonuclease cleaves the probe into individual nucleotides and thus releases the reporter from the quencher. The fluorescence of the reporter can then be detected and increases as each probe is cleaved. The increase in fluorescence is directly related to the amount of cDNA at the start of the reaction. For the different bladder samples tested a relative change in gene expression can be calculated by normalising for the amount of 18S rRNA and generating an arbitrary copy number.

2.6.1 Reagents

The TaqMan® 2x Jump Start Mastermix, Taqman ribosomal RNA control reagent [18s forward primer, 18s reverse primer, 18s probe (VIC reporter dye labelled)], dual labelled oligonucleotide probe (FAM reporter dye labelled) and the Taqman® Gene Expression Assay kits for ENTPDase 1, 2, 3 and 5 were purchased from Applied Biosystems. DNase/RNase free water was purchased from Sigma. All reagents used were thawed and kept on ice during use.

2.6.2 Protocol

The samples to be tested were all of those obtained in the reverse transcription experiment. They were triplicates of each human detrusor sample and the – reverse transcription reaction of each sample. Four non template controls (NTC) were also prepared as controls containing no sample.

A mastermix of the reagents was prepared, sufficient for the number of wells needed.

The volumes and final concentrations of reagents per well were as follows:

2x Jump Start Mastermix	12.5 µl
Reference Dye	0.25 µl
rRNA primer and probe mix	1.25 µl
ENTPDase assay mix (20x)	1.25 µl
DNase/RNase free water	7.25 µl

To each of the wells of the MicroAmp® Optical Reaction 96-well plate 22.5 µl of the mastermix was added. To each of the sample wells 2.5 µl of the sample cDNA was added and to each of the controls 2.5 µl of DNase/RNase free water was added. The mastermix and samples were added quickly and carefully to minimise contamination. MicroAmp® Optical caps were placed securely on the wells of the plate. The plate was then centrifuged (Eppendorf Centrifuge 5810R) for 1 min to ensure the small volume of solution was at the bottom of each well with no bubbles.

2.6.3 TaqMan® RT-PCR

The TaqMan® (ABIprism™ 7700 Sequence Detector) and computer were switched on in that order. The computer program used was SDS 1.9.1. The default settings were checked to ensure they were 'Plate type: single reporter, Instrument: 7700, Sequence detection and Run: real time'. The FAM option was selected on the dye layer and the plate layout set up using 'sample type'. Each well to contain a sample was designated as unknown, those with no sample but water were designated as no template control. The dye layer was then changed to VIC and again the wells were designated with the appropriate sample type. This was to ensure the machine read both the probe for the gene of interest (FAM dye labelled) and the probe for the ribosomal control (VIC dye labelled). The instrument exposure time was set to 25 seconds. The thermal cycler conditions were set at 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The sample volume was set to 25µl and then the run could be

started. Once the run was completed the plate was removed from the instrument and discarded in a biohazard bin.

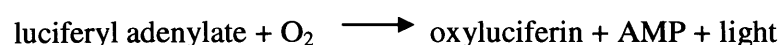
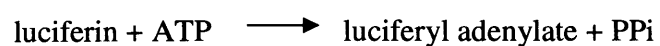
2.6.4 Data analysis

The linear plot of the raw fluorescent data for the FAM dye label from the run was selected. The baseline was moved so that it crossed the first curve just as it began to rise. The ordinate was set to a logarithmic scale to ensure the baseline was within the exponential part of the plot, i.e. the plot appeared linear. The procedure was repeated for data from the VIC dye label run. The plate layout was then shown with the brightness of the wells indicating the final amount of PCR product generated in the particular well. The wells containing samples should have been bright whilst the no template control should be dark. The raw numerical data was exported from the computer. The data was in the form of threshold cycle (C_T) which is the time point expressed in the number of PCR cycles at which the fluorescence signal generated by probe cleavage reaches a detectable threshold. The relative quantity of the gene of interest (FAM) was expressed as a percentage of the house keeping gene (VIC). This was calculated in the following way; for each sample the value of FAM C_T subtracted from VIC C_T was calculated (the number of additional cycles required for FAM to reach the threshold level compared to VIC). The value of 2 to the power of this difference was calculated (every cycle will double the amount of DNA, therefore this represents the fraction of the gene of interest compared to the housekeeping gene), this value was expressed as a percentage of the housekeeping gene.

2.7 ECTONUCLEOTIDASE ACTIVITY

2.7.1 Preparation of ATP assay kit

The ATP assay kit (Sigma) was prepared as described in the instructions. The assay kit contains luciferin and luciferase. Following the addition of the assay kit to a sample containing ATP the following reactions occur, the first is catalysed by luciferase;



The light intensity produced can be measured by a photomultiplier and is proportional to the ATP concentration of the sample.

The assay mix was dissolved in 5 ml of sterile water and frozen at -20°C in 100 µl aliquots. The assay mix dilution buffer was dissolved in 50 ml of sterile water and store at 4°C. The ATP assay mix was diluted 25-fold with the assay mix dilution buffer when used for determining the ATP standard curve and in sample analysis. The diluted mixed was prepared daily.

2.7.2 Preparation of Tissue

Guinea pig bladder strips of approximately 20 mg (dimensions – 3 mm diameter x 4 mm length) were cut and the urothelium was removed. Each strip was cut into two before being incubated to increase the surface area of the tissue sample, in order to aid diffusion

of the ATP into the tissue. Tissue samples were placed in plastic test tubes containing 3 ml of HEPES-buffered Tyrode's solution.

Human detrusor samples were cut to give four pieces, each sample weighing approximately 20-40 mg, and frozen. The frozen samples were then thawed at room temperature in 3 ml of HEPES-buffered Tyrode's solution, in a plastic test tube, prior to the experiment.

2.7.3 Luminometer

The luminometer used was a Lumistar Galaxy (BMG Labtech Ltd, Aylesbury, UK). The Lumistar was connected to a computer with the corresponding dedicated acquisition software written and configured by BMG Labtech Ltd. Samples were measured in 96-well plates (Optiplate, Packard). The luminometer was set to inject 100 µl of the ATP assay mix into each well that contained a sample or standard just prior to the measurement being taken. Each well that contained a sample or standard was measured for ten seconds. To ensure adequate mixing of the sample and ATP assay mix the plate was shaken throughout measurement. The parameters for shaking were set for orbital shaking with a diameter of 3 mm.

2.7.4 ATP standards

A standard curve was performed every day prior to samples being tested. The gain was determined for each set of standards and samples by using the instruments internal gain adjustment. The instrument measures the well containing the standard with the highest ATP concentration several times and sets a gain accordingly.

The standards were prepared by performing a serial dilution of a 10 mM ATP stock solution. Tyrode's solution containing 5 mM EDTA and no added calcium was used for dilution. The standards' concentration range was 0.01 μM to 30 μM .

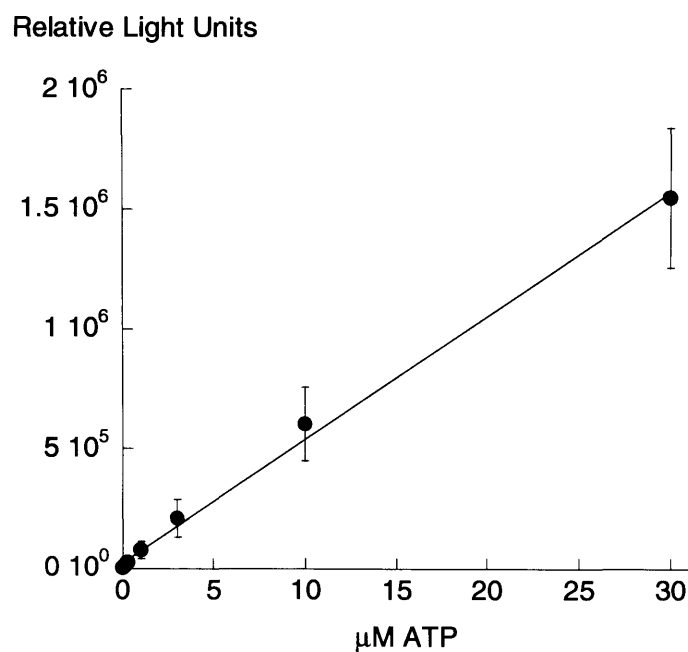


Figure 2.2 Calibration plot of ATP standards. (n=3) Mean ± s.d

2.7.5 Experimental protocol

Two of the four pieces of detrusor cut from each sample of human or guinea pig bladder were used as controls and the other two were used to test the effect of the ectonucleotidase inhibitor ARL 67516.

Both control and test tissue samples were placed in plastic test tubes containing 3 ml of warmed Tyrode's solution. The test tubes were placed in a shaking water bath (Grant SS40-2, Grant Instruments, Cambridge) heated to 37°C and left to equilibrate for approximately 30 minutes. Following equilibration the tissue samples were transferred carefully using tweezers to a tube containing 950-990 µl of either Tyrode's, (control) or Tyrode's with 100 µM ARL 67156, (test). Both solutions had been warmed to 37°C in the water bath. Samples were left for 10-15 minutes shaking in the water bath prior to the addition of one of five concentrations of ATP, in order to measure the rate of ATP breakdown.

At time zero 10, 20 or 50 µl of a stock ATP solution (10 mM or 100 mM) was added to each of the control and test samples to give a final ATP concentration of either 200 µM, 500 µM, 1 mM, 2 mM or 5 mM and in each case a final volume of 1 ml. The samples were left in the heated water bath shaking for 30 minutes. At set time points (0, 5, 10, 20, 30 minutes) 10 µl aliquots of each sample were taken. When investigating the degradation of 200 µM and 500 µM the 10 µl aliquots taken were immediately placed in eppendorf tubes containing 990 µl of chilled calcium-free Tyrode's solution containing

5m M EDTA. During 1, 2 and 5mM ATP degradation two 10 μ l aliquots were taken at each of the five time points and placed in separate eppendorf tubes containing 1.99ml of chilled calcium-free Tyrode's containing 5mM EDTA. Following addition of the 10 μ l aliquots to the eppendorf tubes the samples were vortex-mixed (Genie 2, Scientific Industries) and stored in the fridge for same-day analysis.

Each tissue sample was used to assay the rate of ATP breakdown over the entire range of ATP concentrations, starting with the lowest concentration. In between each ATP concentration the tissue sample was washed in 3.5 ml of fresh warmed Tyrode's solution, in a test tube placed in the shaking water bath for 15 minutes. Following washing, the tissue samples were transferred to 950-990 μ l (depending on the concentration of ATP to be tested) of Tyrode's solution or 100 μ M ARL 67156 Tyrode's solution and incubated for 10 minutes in the water bath prior to the addition of the next ATP concentration to be tested. Once all ATP concentrations had been studied each tissue sample was weighed.

2.7.6 Measurement of ATP.

To determine the rate of degradation of each ATP concentration the diluted aliquots taken from each time-point, at the five ATP concentrations, were assayed for ATP using the luciferin-luciferase assay.

From each of the diluted sample aliquots 100 μ l was pipetted into wells of a white 96-well plate (White 96-well Optiplate, Perkinelmer). The samples were spaced out on the

plate, in order to limit the effect of cross-over between wells affecting the recorded measurement.

2.8 AMPEROMETRIC ATP DETECTION

2.8.1 Experimental set up - tissue

Guinea pig bladder was the tissue used in experiments measuring the release of ATP from detrusor. The bladder strips were prepared following the same method as those in the tension experiments (see section 2.3). The one difference was the size of the strip. As larger strips were required they were cut slightly wider and ran the whole length of the bladder, from base to dome. The strips were approximately 2 mm wide and between 7-10 mm in length.

The strips were secured into the horizontal organ bath and superfused with Tyrode's solution containing 2 mM glycerol and was constantly gassed with 95% O₂, 5% CO₂ mixture and warmed to 37°C. The flow rate of the superfusate was 2 ml.min⁻¹. The strips were lengthened using micromanipulators until a maximal contraction was achieved whilst ensuring minimal tissue movement.

All solutions used for interventions were warmed to 37°C in the water bath and gassed with a 95% O₂, 5% CO₂ mixture. All solutions were prepared with Tyrode's solution containing 2 mM glycerol.

2.8.2 Experimental set up - biosensors

The ATP biosensor (Sarrisaprobe™-ATP, Sarrisa Biomedical Ltd, Coventry, UK) electrode is a needle shaped microelectrode constructed of Pt/Ir wire that is coated at the end with a polymer containing specific enzymes. The electrode specifications are shown in Table 2.4. In the presence of glycerol ATP is utilised by the enzymes to give a final product of H₂O₂ which can be detected amperometrically by the electrodes. The null electrode (SA10002, Sarrisa Biomedical Ltd, Coventry, UK) is essentially the same as the ATP biosensor except that the polymer coat lacks any enzymes.

Table 2.4 ATP Biosensor specifications

Sensor length	(tip) 2 mm x 50 µm (diameter)
Response time	10-90% rise time ≤ 10 seconds
Linear range	0.5 µM to 50 µM
Sensitivity	0.5 nA per µM

Both ATP and null electrodes were purchased dehydrated and were stored at 4°C. Rehydration required the electrode tip to be immersed in Tyrode's solution containing 2 mM glycerol for approximately 10 minutes. Once hydrated it was important to minimise

exposure of the electrodes to air. Hydrated electrodes could be stored in Tyrode's solution at 4°C and used for up to 5 days. Due to reduction in electrode sensitivity the electrodes were discarded after two sets of experiments.

Prior to rehydration, the electrodes were carefully bent 80° to enable them to lie across the top of the tissue. They were bent at the point where the Pt/Ir wire becomes visible and the enzyme-containing plastic coating ends.

The ATP and null biosensors were connected to a potentiostat (MicroC low-noise carbon fibre potentiostat, World Precision Instruments Ltd, Aston, UK) via Triax shielded cables. The potentiostat specifications are listed in Table 2.5.

A Ag/AgCl reference electrode was placed in a 3 M-KCl salt bridge, the tip of which was immersed in the superfusate solution in the bath. The reference electrode was connected to the two potentiostats to act as a reference for both the ATP and null biosensors. This electrode was at the same ground potential for the bath and measuring equipment.

Table 2.5 Potentiostat specifications

Electrode polarisation voltage	0.65 V (adjustable from 0 to 2 μ A)
Current ranges	2000 pA, 20 nA, 200 nA, 2 μ A
Current/voltage conversion factor	From 1 pA.mV ⁻¹ to 1 nA.mV ⁻¹
Rise time	1 millisecond

Micromanipulators were situated on the table next to the bath and were positioned appropriately prior to mounting the sensors so as to reduce the risk of air exposure and damage. Once the ATP and null biosensors were inserted into the holders they were quickly lowered into the bath so that they were submerged in the superfusate. With the use of a binocular microscope the biosensors were positioned side-by-side in the superfusate above the tissue as far from the embedded platinum stimulating electrodes as possible. This was to minimize the effect of a stimulation artefact on the recording.

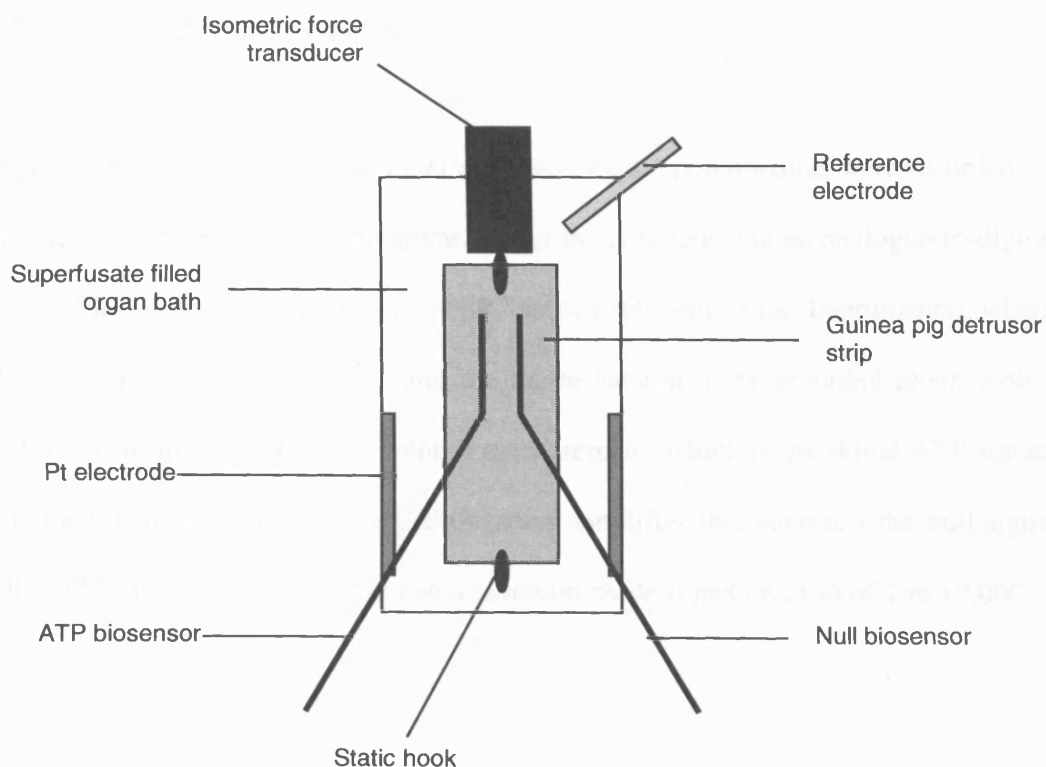


Figure 2.3 Diagram showing the positioning of guinea pig detrusor muscle and biosensors used for measuring the release of ATP.

When the biosensors were in a satisfactory position the potentiostats were switched on and the sensors began polarizing. It could take up to 10 minutes for polarization to take place. The polarization potential was set at 500 mV.

2.8.3 Experimental set-up – recording

The signals produced by the sensors and measured by the potentiostats were recorded on a computer. The potentiostats were connected to the computer via an analogue-to-digital converter. The software program used was Clampex (pClamp Axon Instruments), which enabled the two biosensors signals and the tissue tension to be recorded along with a differential measurement. This differential measurement, which is the actual ATP signal, was attained using a home-made DC differential amplifier that subtracts the null signal from the ATP signal. The amplifier had a common mode rejection ratio of 1 in 10,000.

2.8.4 Biosensor calibration

Calibration of the electrode was performed at the beginning and end of each experiment and at least once during the experiment to ensure electrode sensitivity was satisfactory.

The calibration was performed in situ when 10 μ M ATP in 2 mM glycerol Tyrode's solution was gassed with 95% O₂ 5% CO₂ and superfused to the tissue and sensors, a sample is shown in Figure 2.4. The electrodes display a linear response to ATP over the concentration range 200 nM to 50 μ M (Llaudet et al, 2005) and therefore a one point calibration is recommended by the manufacturers.

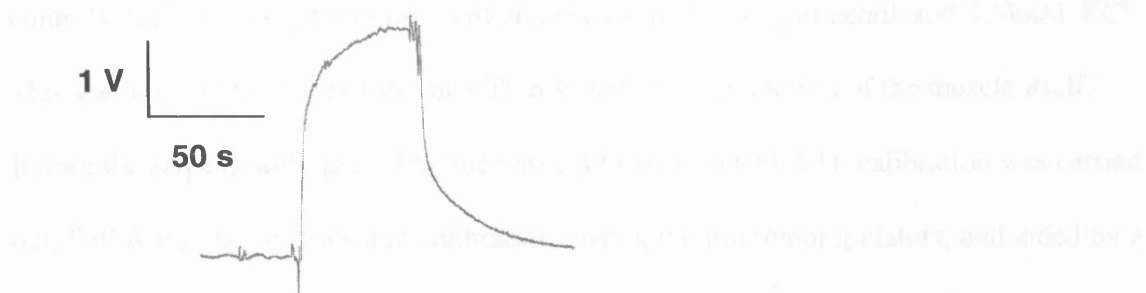


Figure 2.4 Sample calibration recording for 10 μ M ATP

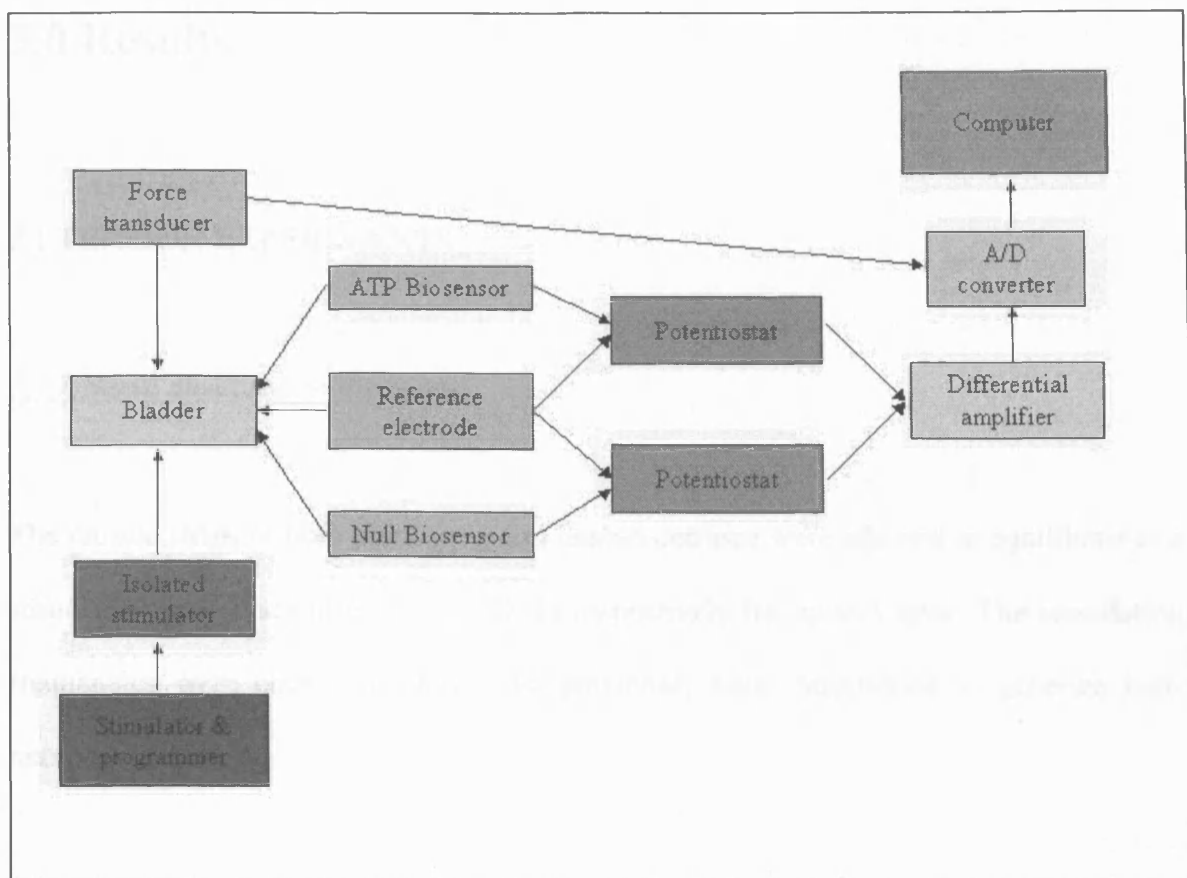
2.8.5 Experimental protocol

Before the sensors were positioned the tissue was stimulated at 8 Hz until isometric phasic tension reached a steady level. The stimulation parameters were the same as those described for the tension experiments (see section 2.3) except the interval between stimulation trains was 110 seconds. An optically-isolated constant-voltage stimulator (DS2 Digitimer, Hertfordshire, UK) to minimise pick-up of stimulation artefacts was connected between the stimulator and platinum electrodes in the bath. The output voltage was adjusted to achieve a satisfactory steady level contraction of the tissue and minimal stimulation artefact on the sensor recordings. The stimulator was switched off whilst the sensors were being placed on the preparation and subsequently polarized.

Following polarization a 10 μ M ATP calibration was performed. If successful the experiment continued with a set of controls whereby the electrodes remained in the superfusate, off the tissue. The first control was the generation of a force-frequency curve by stimulating the strips at a range of frequencies, between 1 and 24 Hz. The other

controls consisted of generating contractures using 3 μ M carbachol and 120mM KCl. This was to determine the extent of ATP release from contraction of the muscle itself. Before the sensors were placed on the tissue a further 10 μ M ATP calibration was carried out. Following the controls and calibrations, using the micromanipulators, and aided by a binocular dissection microscope, the sensors were placed on the tissue. The best results were obtained when the sensors were lowered sufficiently so that they were slightly embedded in the tissue and no longer visible. The electrodes were often repositioned a number of times in order to obtain a response that was not effected by movement of the muscle. The sensors were placed as far from the Pt stimulating electrodes were positioned close together to reduce interference.

With the sensors in place a force-frequency curve was generated to measure release of ATP at the various stimulation frequencies (1-24 Hz) and the 3 μ M carbachol and 120 mM KCl contractures were repeated. The effect of 100 μ M atropine, ABMA and nifedipine, individually or combined, on the ATP released during the force-frequency curve or at fixed frequencies was examined. A force-frequency curve in the presence of TTX was always generated at the end of each experiment, followed by a final 10 μ M ATP calibration.



Scheme 2.1 Block diagram of the set-up for measuring ATP release from guinea pig detrusor strips.

2.9 DATA PRESENTATION AND STATISTICAL ANALYSES

Data sets are presented at means \pm standard deviations, unless otherwise stated. Data from the RT-PCR measurements are expressed as medians and 25%, 75% interquartiles because these are derived from logarithmic transformations of the primary measurements. To test for significance between data sets, paired or unpaired Student's *t*-tests were used for mean data sets. For median data sets a Mann-Whitney U-test was used. The null hypothesis was rejected when $p > 0.05$.

3.0 Results

3.1 TENSION EXPERIMENTS

3.1.1 Nerve mediated contractions

The muscle strips of both guinea pig and human detrusor were allowed to equilibrate at a stimulation frequency of 8 Hz and 20 Hz respectively for up to 1 hour. The stimulation frequencies were chosen as they have previously been determined to generate half-maximal tension.

The mean maximum nerve-mediated force estimated from force-frequency curves by guinea pig from the force-frequency was $27.7 \pm 17.4 \text{ mN.mm}^{-2}$ (n=57). The mean tension at 8 Hz ($11.9 \pm 7.2 \text{ mN.mm}^{-2}$) was $43 \pm 19.5\%$ of the maximum force. The mean half maximal frequency ($f_{1/2}$) for guinea pig strips was $9.5 \pm 3.5 \text{ Hz}$.

For the stable human detrusor strips the estimated mean maximum force generated during the force frequency was $27.2 \pm 20.4 \text{ mN.mm}^{-2}$ (n=25). The mean tension at 20 Hz was $11.7 \pm 5.4 \text{ mN.mm}^{-2}$. For this group of samples the mean $f_{1/2}$ was $15.9 \pm 7.0 \text{ Hz}$.

For the combined overactive human detrusor group (n=28) the estimated mean maximum force generated was $24.3 \pm 15.4 \text{ mN.mm}^{-2}$ with a mean $f_{1/2}$ of $19.9 \pm 11.2 \text{ mN/mm}^2$. In this group the mean tension at 20 Hz was $14.6 \pm 13.2 \text{ mN.mm}^{-2}$.

The estimated maximum force values, $f_{1/2}$ values and mean tension achieved at 20 Hz were not significantly different between the stable and overactive human groups.

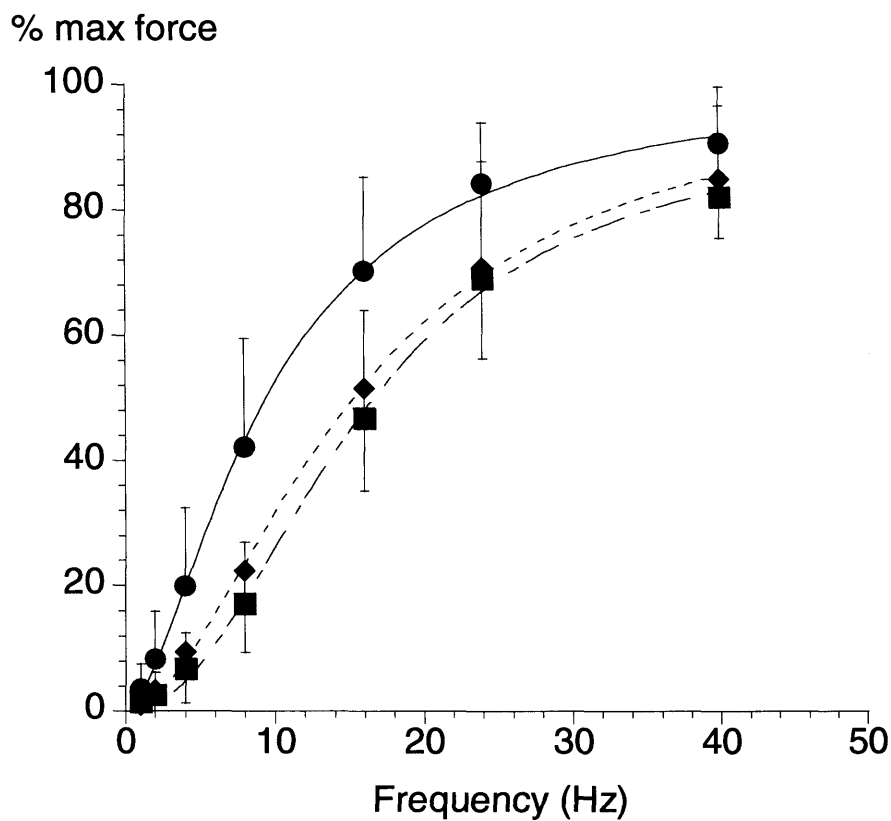


Figure 3.1 Force frequency curves for guinea pig and human stable and overactive detrusor strips as percentage of estimated maximal tension; Guinea pig, circles: stable human, diamonds: overactive, squares. Mean values \pm s.d.

3.1.2 Atropine resistance

At the end of the tension experiments guinea pig and human tissue was stimulated at 8 Hz and 20 Hz respectively in the presence of the muscarinic antagonist atropine (1 μ M). This was followed by the addition of tetrodotoxin (TTX) which blocks the release of neurotransmitters from the nerve. In the presence of TTX contractions were abolished indicating there was no direct muscle stimulated component of contraction. The atropine resistant component was calculated as the TTX-sensitive contraction after the addition of atropine.

In guinea pigs, two neurotransmitters initiate contraction, acetylcholine (Ach) and ATP (Bayliss et al, 1999). Atropine blocks the Ach-dependent component. Force-frequency curves were generated in the presence of atropine and the mean maximum force was reduced to 41.9% of that in control conditions ($12.9 \pm 6.95 \text{ mN.mm}^{-2}$). The mean $f_{1/2}$ value, $14.06 \pm 11.84 \text{ Hz}$, was not significantly different from that in the presence of atropine. The mean tension generated at 8Hz was also reduced to $5.03 \pm 3.26 \text{ mN.mm}^{-2}$.

When atropine was added to the stable human preparations the contractions were completely abolished in all but three of the 25 strips. The lack of atropine resistant contractions in human stable bladder samples agrees with previous observations. With the three preparations where atropine resistance was observed the donor patients did not have urodynamic assessments prior to operation and for the reason categorised as stable.

In contrast to samples from stable human bladders, atropine-resistant contractions have been demonstrated in some bladder pathologies (Bayliss et al, 1999). This nerve-mediated atropine resistance could be eliminated by desensitising the P2X receptors with ABMA, implying the contraction was activated by ATP. In the combined over-active human group 11 of the 28 samples showed some atropine resistance. In the presence of atropine the median maximum force generated was reduced to 13.8% of that in control conditions. The mean $f\frac{1}{2}$ was 13.8 ± 6.4 Hz, as shown in Figure 3.4 was significantly reduced in the presence of atropine. The percentages of atropine resistance for the samples of each group are shown in Figure 3.2 and force frequency curves in guinea-pig and overactive human detrusor samples in the presence of atropine are shown in Figure 3.3.

% atropine resistance

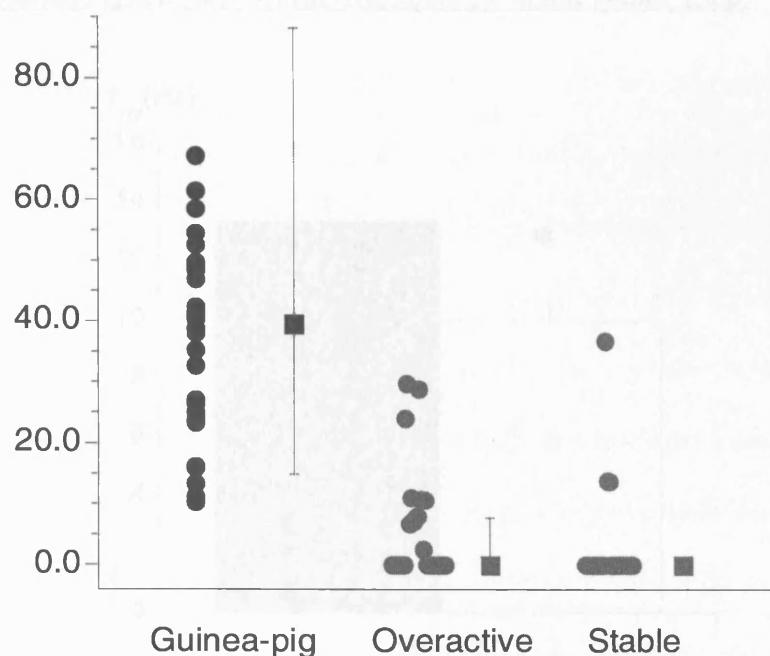


Figure 3.2 Percentage of atropine resistance for each guinea-pig, stable and overactive human detrusor samples (circles). Median and 25th and 75th quartiles (squares).

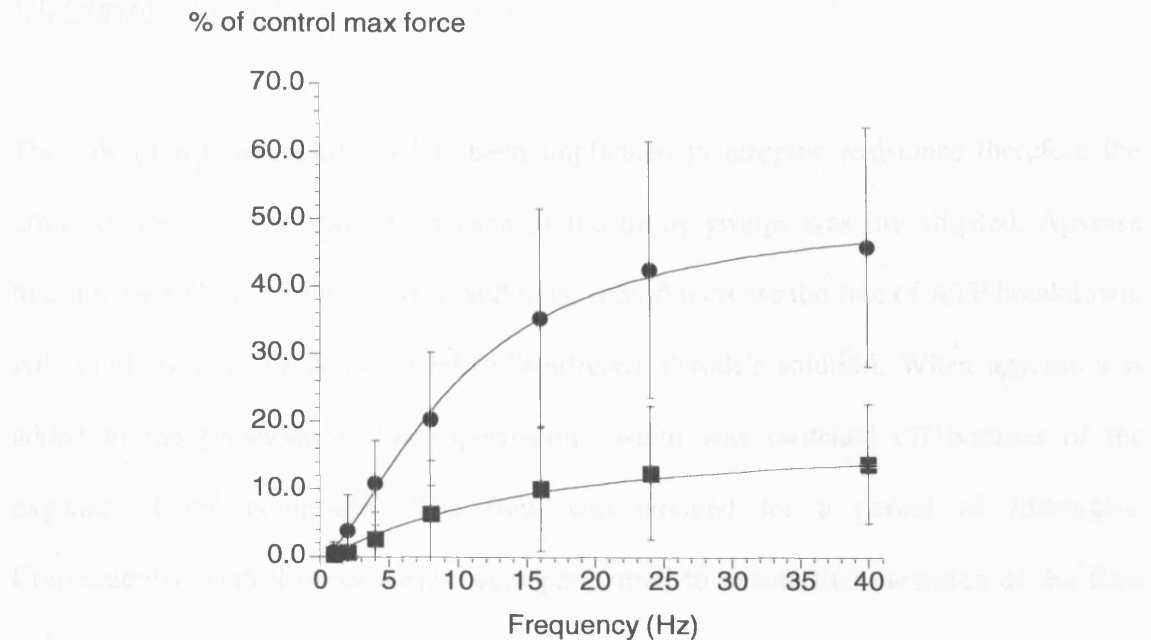


Figure 3.3 Force-frequency plot for guinea pig and over active detrusor strips in the presence of 1 μ M atropine as percentage of estimated maximal tension in control conditions; Guinea pig, circles; overactive, squares. Mean values \pm s.d.

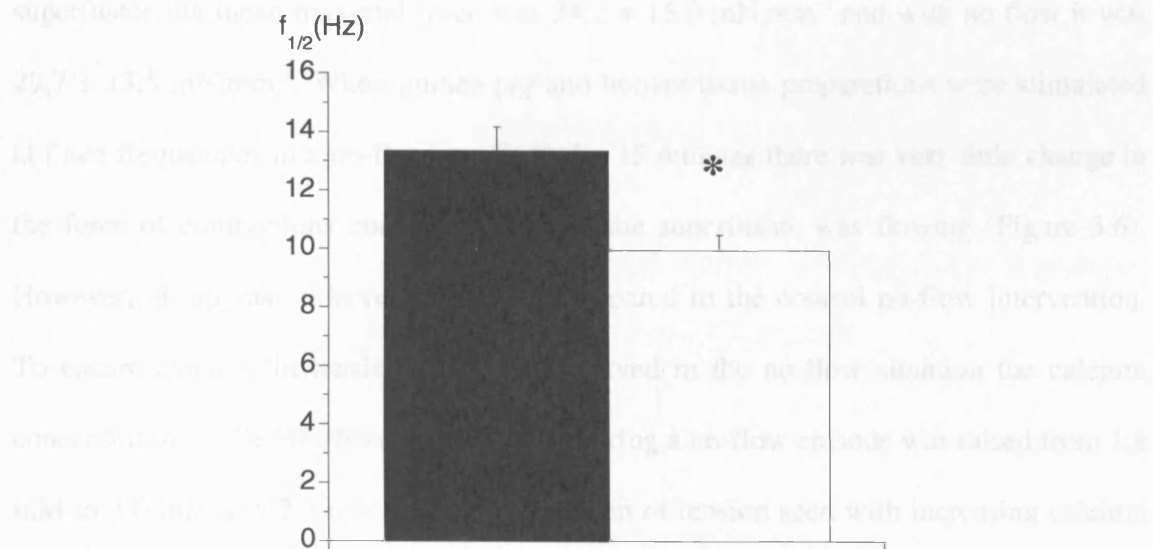


Figure 3.4. Estimated $f_{1/2}$ values for overactive human detrusor in the presence (white) and absence (black) of 1 μ M atropine; $n=11$, mean values \pm s.d.

3.1.3 Apyrase experiments

The role of ectonucleotidases has been implicated in atropine resistance therefore the affect of the enzyme apyrase on each of the tissue groups was investigated. Apyrase hydrolyses ATP and ADP to AMP and thus, should increase the rate of ATP breakdown. All solutions were prepared in HEPES-buffered Tyrode's solution. When apyrase was added to the preparations the superfusion system was switched off because of the expense of the compound. The flow was stopped for a period of 15minutes. Consequently control experiments were performed to ensure that cessation of the flow did not have an effect on the contractile parameters.

Force frequency curves were constructed in flow and no-flow conditions (n=19). Figure 3.5 shows that the force frequency relationships were not significantly different. With superfusate the mean maximal force was $34.2 \pm 15.6 \text{ mN.mm}^{-2}$ and with no flow it was $29.7 \pm 13.5 \text{ mN.mm}^{-2}$. When guinea pig and human tissue preparations were stimulated at fixed frequencies in a no-flow condition for 15 minutes there was very little change in the force of contractions compared to when the superfusate was flowing (Figure 3.6). However, all apyrase interventions were compared to the control no-flow intervention. To ensure changes in tension could be observed in the no-flow situation the calcium concentration of the HEPES solution added during a no-flow episode was raised from 1.8 mM to 3.6 mM and 7.2 mM. The augmentation of tension seen with increasing calcium concentration when the solution was flowing, was the same as when the flow was stopped. Therefore, the cessation of flow for a brief interval did not *per se* affect the

contractile properties of the preparation or their ability to respond to inotropic intervention.

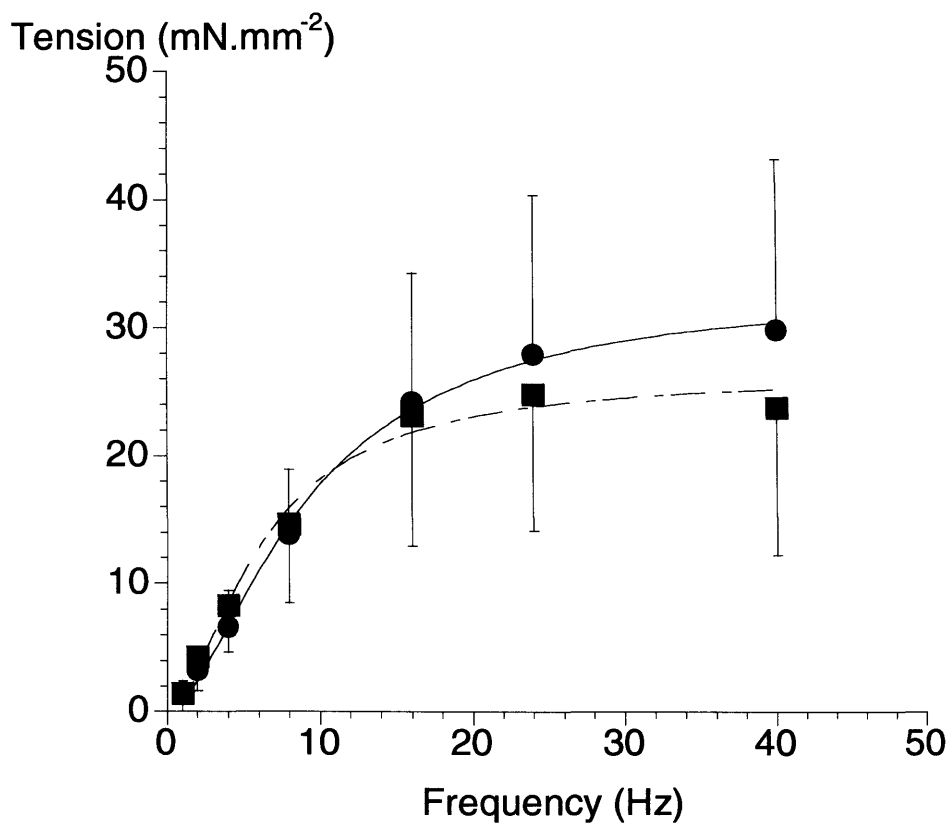


Figure 3.5 Force-frequency curves of guinea pig detrusor preparations in superfusate flow (circles) and no-flow (squares) conditions, mean values \pm s.d.

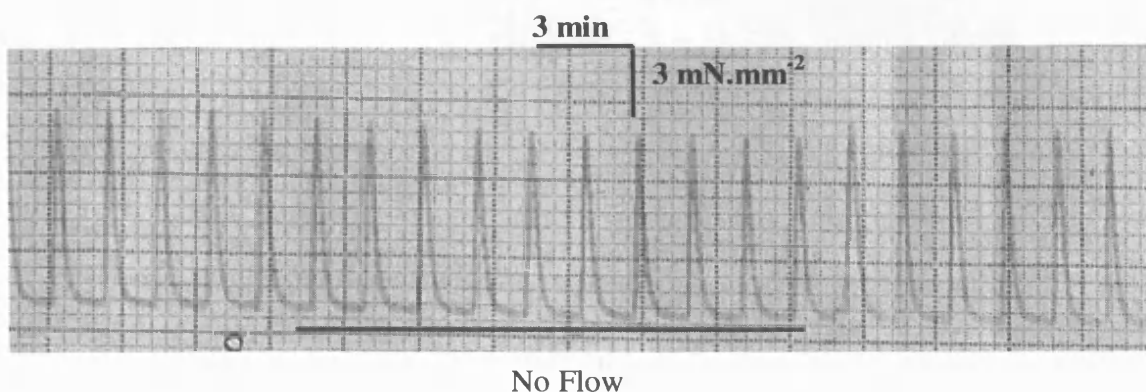


Figure 3.6 Sample trace of guinea pig detrusor preparation during a no-flow condition for a 15 minute period. Tissue was stimulated at 8 Hz.

3.1.4 Apyrase experiments –guinea pig detrusor

Apyrase (10 U.ml^{-1}) reduced significantly tension elicited at 16 Hz stimulation when compared to control. This effect of apyrase was completely and rapidly reversed on return to control solution (see Figure 3.7). In 21 experiments the tension was reduced to $81.5 \pm 14.5 \%$ of control. Apyrase also caused similar significant reductions of tension when the frequency was fixed at 2, 4 and 8 Hz when compared to control. These results are summarised in table 3.1.

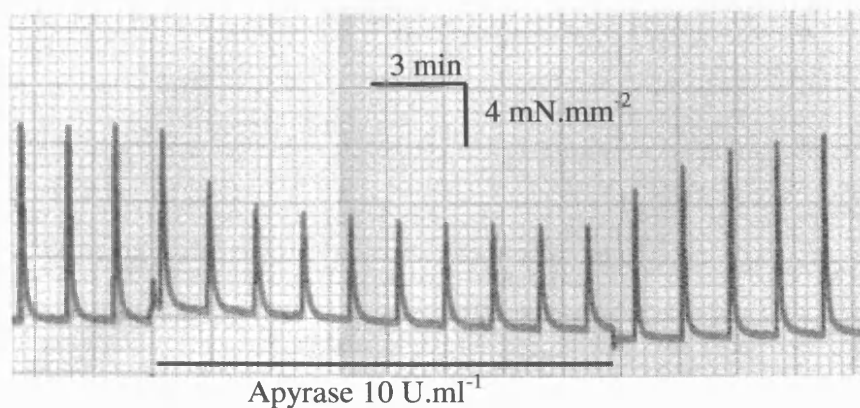


Figure 3.7 Sample trace showing the effect of apyrase on 16 Hz nerve-mediated contraction of guinea pig detrusor. Apyrase was present for 15 minutes.

3.1.5 Apyrase experiments –human detrusor; stable bladders

Apyrase had no significant effect on the contraction elicited by 24 Hz stimulation ($n=12$), Figure 3.8. The mean tension achieved at 24 Hz stimulation in apyrase was $103 \pm 16\%$ of that in control. Preparations were stimulated at 24 Hz as this frequency is just above the estimated $f_{1/2}$ for this tissue.

The lack of effect of apyrase is consistent with the hypothesis that there is no ATP component of contraction in human detrusor from stable bladders.

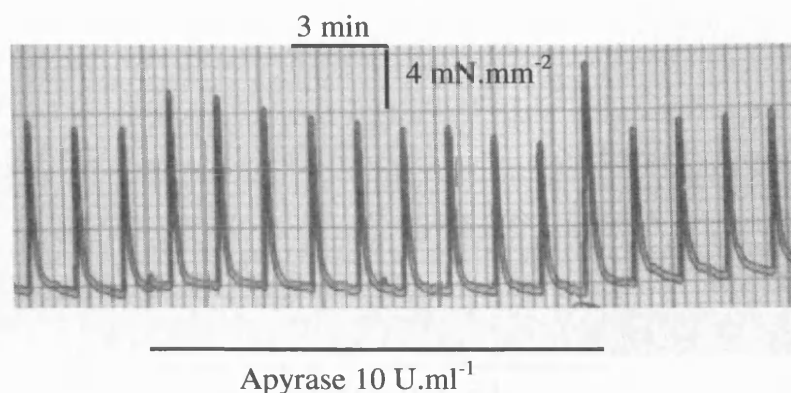


Figure 3.8 Sample trace showing the effect of apyrase on 24 Hz nerve-mediated contraction of stable human detrusor

3.1.6 Apyrase experiments –human detrusor; overactive bladders

The human preparations from overactive bladders were stimulated at 24 Hz in the presence of apyrase for 15 minutes. The result was a significant reduction in the tension generated, Figure 3.9. The mean tension was decreased to 87.5 ± 7.5 % compared to control (n=14). When the frequency was fixed at 4, 8, 16 and 20 Hz apyrase caused a reduction in the tension generated however the reductions were not significant. This may be due to the low sample numbers. In all experiments where there was a reduction, contractions returned to pre-apyrase tension upon return to HEPES solution.

As with the guinea pig preparations the reduction caused by apyrase in the overactive strips indicates that a proportion of the nerve-mediated contraction is initiated by ATP and that by increasing its hydrolysis the contraction tension can be reduced.

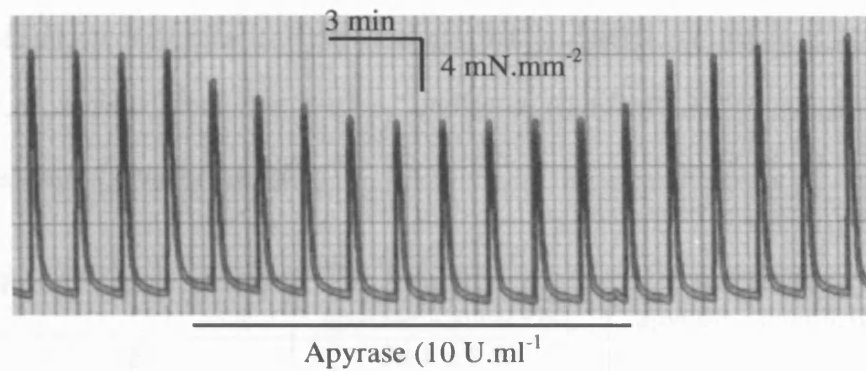


Figure 3.9 Sample trace showing the effect of 10 U.ml⁻¹ Apyrase on 24 Hz nerve-mediated contraction of human detrusor from overactive bladders

The percentage reduction caused by apyrase on bladder pressure during nerve-mediated contraction of guinea pig ($n = 10$) and human detrusor ($n = 10$) is summarized in Table 3.10a. Apyrase in both guinea pig and overactive human detrusor caused a significant with a greater reduction in the guinea pig bladder. This is due to the large ATP-dependent contraction of the guinea pig bladder. The reduction in overactive human detrusor is significant (Figures 3.11 and 3.12) compared to the control (Figure 3.10) and is due to the large ATP-dependent contraction of the overactive human detrusor. The reduction in overactive human detrusor is significant (Figures 3.11 and 3.12) compared to the control (Figure 3.10) and is due to the large ATP-dependent contraction of the overactive human detrusor.

Table 3.1 Summary of results of effect of apyrase in guinea pig and over active human detrusor at various fixed frequencies.* indicates significant difference from control, $p<0.05$ unpaired Student's t-test

Fixed Frequency (Hz)	% reduced to by apyrase – guinea pig	% reduced to by apyrase – over active
2	80.1 \pm 8.3* (n=6)	-
4	78.7 \pm 11.2* (n=8)	97.4 \pm 13.6 (n=2)
8	79.9 \pm 14.3* (n=11)	95.7 \pm .3 (n=4)
16	81.5 \pm 14.5* (n=21)	95.3 \pm 6.4 (n=5)
20	-	90.1 \pm 17.2 (n=11)
24	-	87.5 \pm 7.5* (n=14)

The percentage reductions caused by apyrase on fixed-frequency nerve-mediated contractions of guinea pig (16 Hz) and human detrusor (24 Hz) are summarised in Figure 3.10a. Reduction in both guinea pig and overactive human detrusor groups were significant with a greater attenuation in the guinea pig samples. This is in concord with the larger ATP-dependent component of contraction in guinea pig compared to overactive human detrusor. Comparing Figure 3.10a and 3.10b (reprint of Figure 3.2 for comparison with Figure 3.10a) shows that atropine and apyrase have the greatest effect in guinea-pig detrusor, little or no effect in detrusor from stable bladders and an intermediate effect in detrusor from overactive human bladders.

% Reduction by apyrase

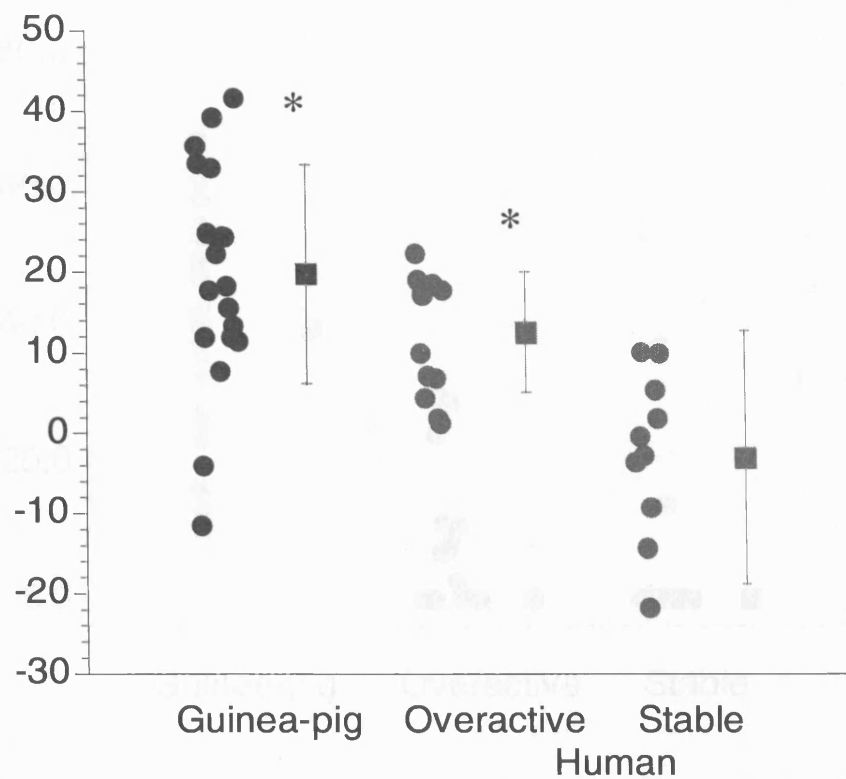


Figure 3.10a Effect of apyrase on fixed frequency nerve-mediated contractions in detrusor from guinea pig (16 Hz)(blue) and overactive (red) or stable (green) human bladders (24 Hz). Mean \pm standard deviation (squares), * indicates significant difference from control, $p < 0.05$ unpaired student's t-Test

3.1.7 ARL 67156 effects on guinea pig detrusor

An alternative way of manipulating ectonucleotidase activity in detrusor samples was to inhibit activity using the ectonucleotidase specific inhibitor ARL 67156 (100 μ M). A concentration of 100 μ M was used as this concentration was used previously to inhibit ATP action whilst not effecting P2X receptors (Crack *et al*, 1995; Westfall *et al*, 1997). The trace in Figure 3.11 shows the effect of 100 μ M ARL 67156 on a guinea pig strip stimulated at 16 Hz. The inhibitor caused a rapid increase of tension which was completely reversible upon return to Tyrode's solution.

In a number of experiments (n=6) 100 μ M ARL 67156 caused a significant average increase in tension of 28.6 ± 20.3 % compared to control as shown in Figure 3.12. With the inhibition of ectonucleotidase activity and therefore a reduced breakdown of ATP there is an increase in contraction.

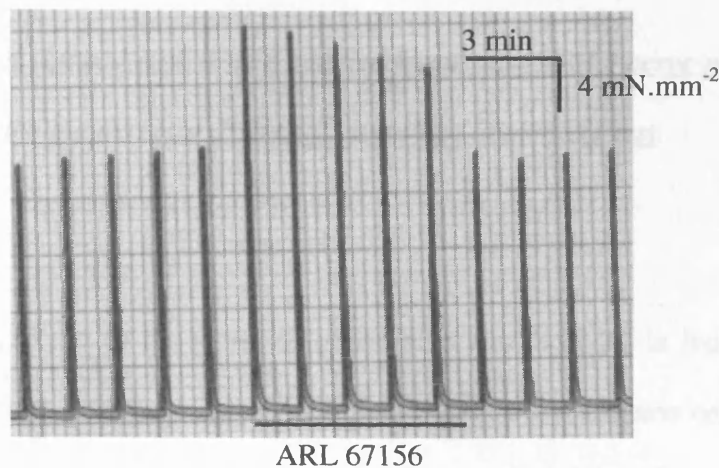


Figure 3.11. Sample trace showing the effect of 100 μ M ARL 67156 on 16 Hz nerve mediated contraction of guinea pig detrusor

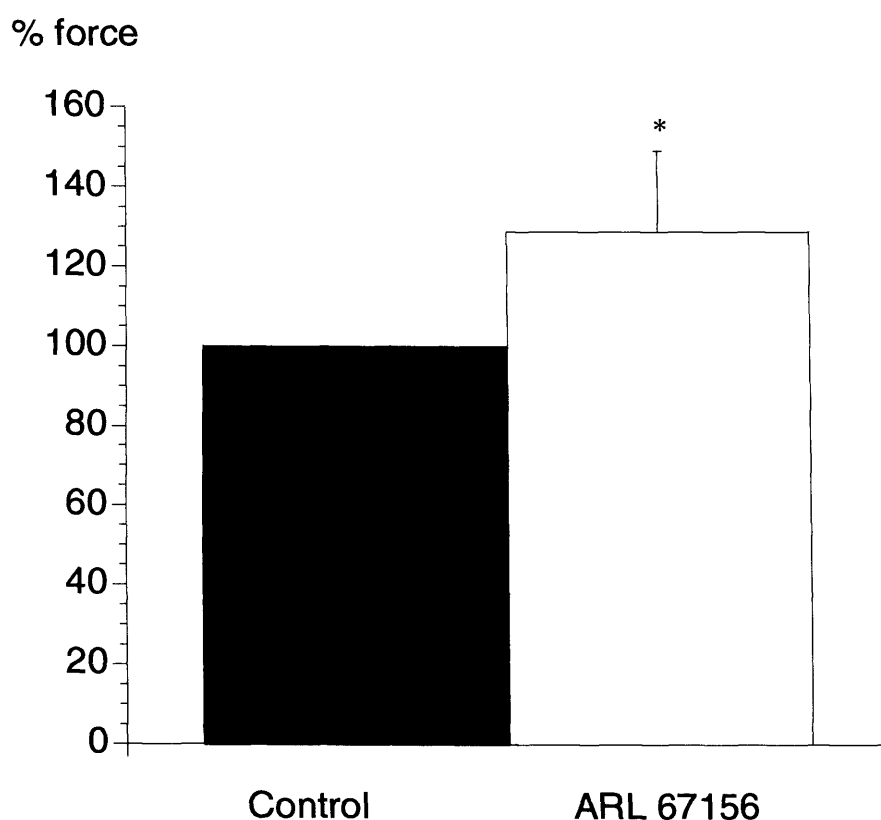


Figure 3.12 Effect of 100 μ M ARL 67156 on fixed frequency (16 Hz) nerve-mediated contractions of guinea pig detrusor ,* $p<0.05$ unpaired Student's t-test

The effect of ARL 67156 in the presence of atropine was recorded in four samples to observe the effect of the inhibitor on the ATP component of contraction only. In Figure 3.13 the control bar is the contraction in the presence of atropine alone. When ARL 67156 was added mean tension significantly increased by 19.0 ± 6.8 %. This further

implied that the increase of tension was due to an increase of the ATP component of contractile activation.

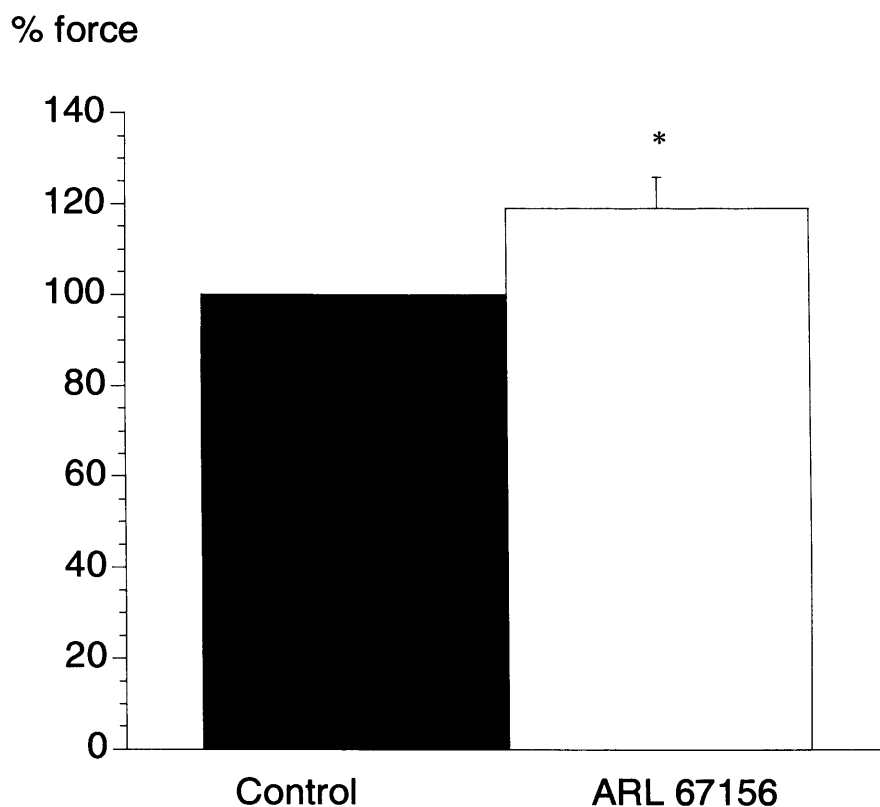


Figure 3.13 Effect of 100 μ M ARL 67156 on fixed frequency (16Hz) nerve-mediated contractions in the presence of atropine in guinea pig detrusor. Control: black, ARL 67156: white., * $p < 0.05$ unpaired Student's t-test

3.1.8 Summary of results

In guinea-pig and human detrusor from overactive bladders increasing addition of apyrase, and thus increasing ATP breakdown, reduced the force of contraction. This was

unlike in detrusor from stable human bladders where apyrase was without effect. The lack of atropine resistance and effect of apyrase in human samples from stable bladders is consistent with the hypothesis that breakdown of ATP released due nerve stimulation is complete. Atropine resistance and an apyrase effect in samples from overactive human bladders indicate an incomplete degradation following nerve stimulated release. Inhibition of ectonucleotidase in guinea-pig detrusor by ARL 67156 resulted in an increase in contraction force. These results show that modulating ectonucleotidase activity, and therefore ATP breakdown, can alter the force of nerve mediated contraction in detrusor smooth muscle when there is an atropine resistant component to contraction.

3.2 TOTAL RNA ISOLATION

3.2.1 Extraction and assessment

After extraction of RNA from human detrusor samples using the RNAeasy kit the RNA samples were analysed to determine if they were suitable for use in further gene expression studies. Preparation of RNA can result in its degradation and introduce contaminants, therefore the condition of the RNA has to be established. This was achieved using the Aligent 2100 Bioanalyser and the RNA 6000 Labchip kit which checked for both integrity and purity. The technique used electrophoresis to separate the RNA transcripts according to their mass and an intercalating dye to detect the RNA. All data were recorded by computer.

On each chip a standard ladder was run with fragments of 0.2, 0.5, 1, 2, 4 and 6 kbases. The gel-like image of the ladder can be seen in lane one of each of the gels in Figures 3.15 to 3.17. An example of the electropherogram for the ladders is shown in Figure 3.14. The ladder should appear as six discrete bands on the gel-like images and six peaks on the electropherogram. A successful sample should appear as two discrete bands, one for each of the ribosomal 18s and 28s. The sample electropherogram should show two separated peaks. Each ladder and sample also contains a marker which appears as the bottom band on the gel and the first peak on the electropherogram to indicate that a sample was loaded. The experimental group (i.e. from stable or overactive bladders) of the different sample numbers is shown in Table 3.2 at the end of this section.

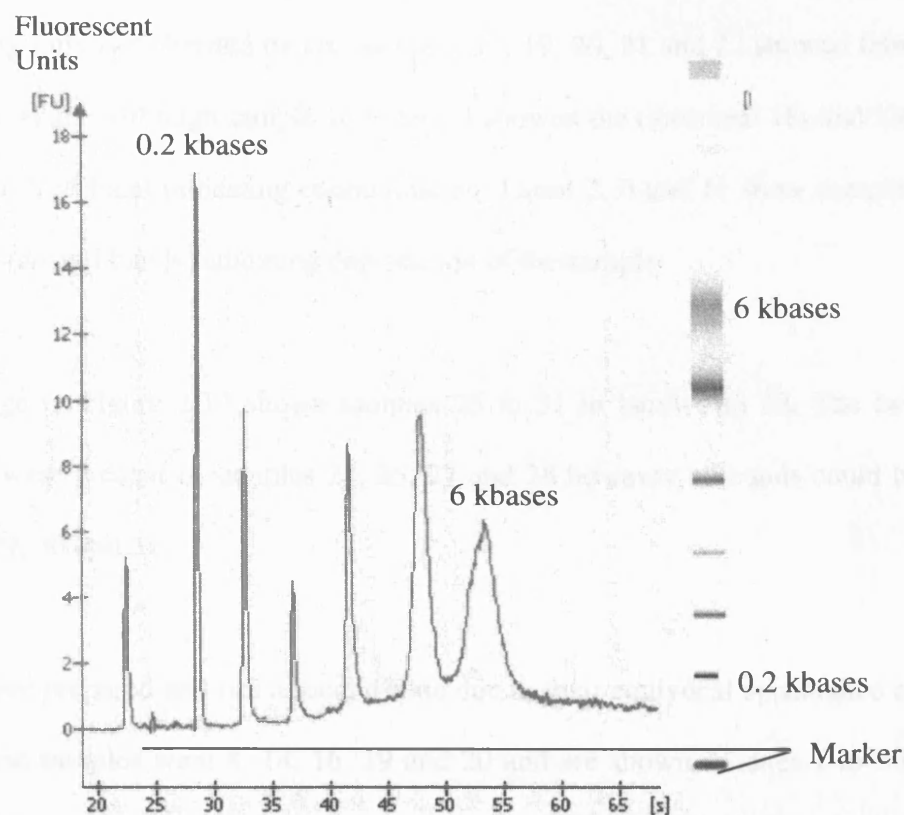


Figure 3.14 Electropherogram showing an RNA 6000 standard ladder which has fragments of 0.2, 0.5, 1, 2, 4 and 6 kbases eluted over time (seconds). The gel-like image of the ladder is shown on the right hand side.

The gel-like image for each of the samples can be seen in Figure 3.15 to 3.17. The gel in Figure 3.15 shows the results for samples A, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11. All samples except 8 and 9 in lanes 4 and 2 respectively showed discrete ribosomal 18s and 28s bands. Sample 8 showed very faint bands whilst no band could be observed for sample 9.

Samples 13 to 24 are shown on the gel-like image in Figure 3.16 with only samples 13, 15 and 24 showing only two discrete bands. Samples 17, 19, 20, 21 and 22 showed faint bands or no bands at all. Although sample 16 in lane 4 showed the ribosomal 18s and 28s bands there was a third band indicating contamination. Lanes 2, 6 and 11 show samples 14, 18 and 23 as smeared bands indicating degradation of the sample.

The gel-like image in Figure 3.17 shows samples 25 to 31 in lanes 6 to 12. The two ribosomal bands were present in samples 25, 26, 27 and 28 however no bands could be seen in samples 29, 30 and 31.

Some samples were prepared and run a second time due to their equivocal appearance on the first run. These samples were 8, 14, 16, 19 and 20 and are shown in lanes 1 to 5 in Figure 3.17. Of the repeated samples only sample 19 showed two discrete bands.

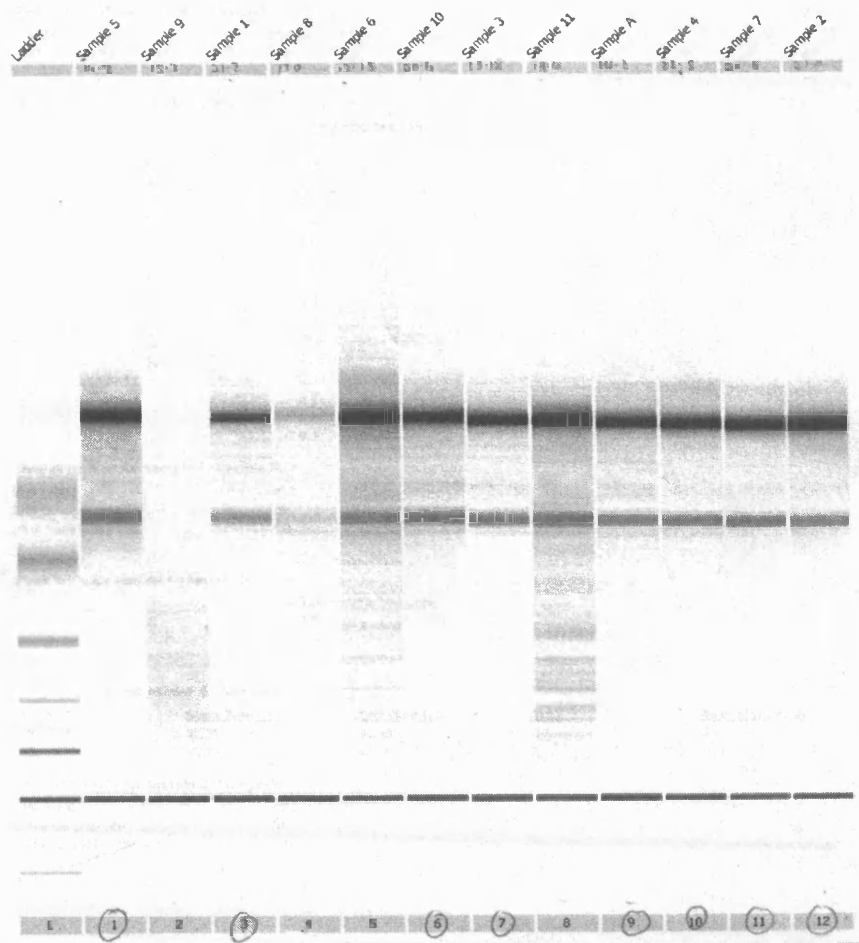


Figure 3.15 Gel like image of total RNA extracted from human detrusor samples. Lane L (far left) shows the RNA 6000 ladder, lanes 1 to 12 show detrusor samples A to 11.

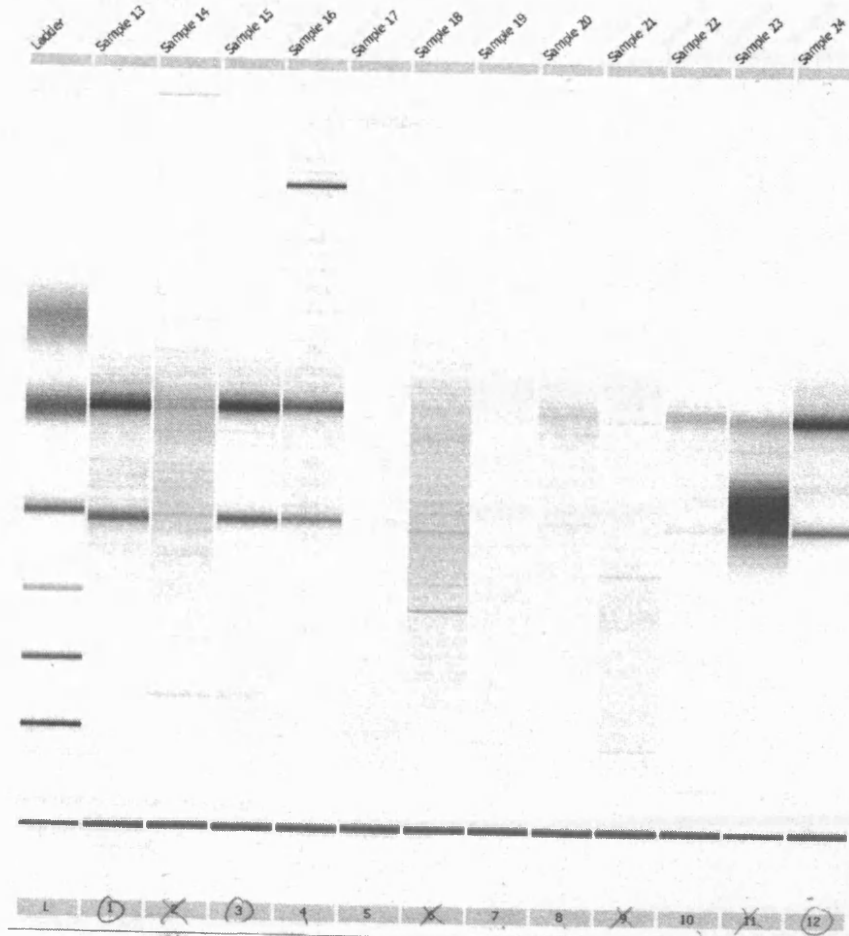


Figure 3.16 Gel-like image of total RNA extracted from human detrusor samples. Lane L shows the RNA 6000 ladder, lanes 1 to 12 show detrusor samples 13 to 24.

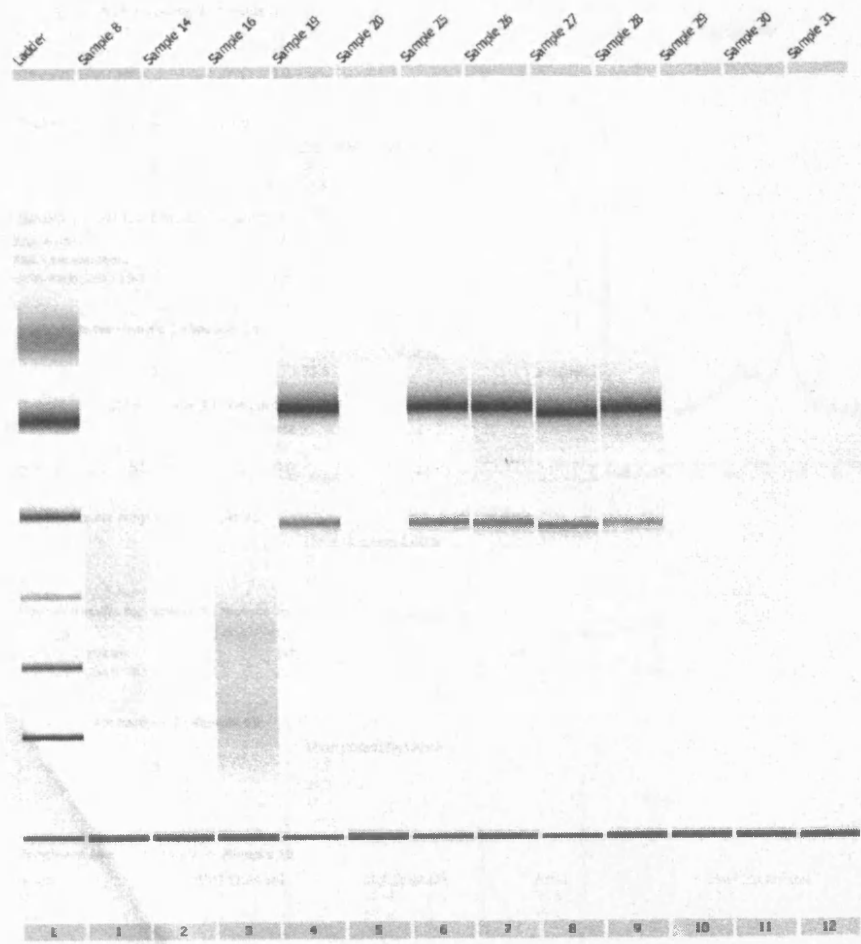


Figure 3.17 Gel-like image of total RNA extracted from human detrusor samples. Lane 1 shows the RNA 6000 ladder, lanes 6 to 12 show detrusor samples 25 to 31 and lanes 1 to 5 show repeats of samples 8, 14, 16, 19 and 20.

The decision to include or exclude an RNA sample in further studies was determined by the appearance on the gel and the resolution of the two ribosomal, 18s and 28s, fluorescent peaks. Figure 3.18 shows electropherogram examples of: i) a non-degraded sample with no contamination; ii) a degraded sample; iii) a sample with a contaminate.

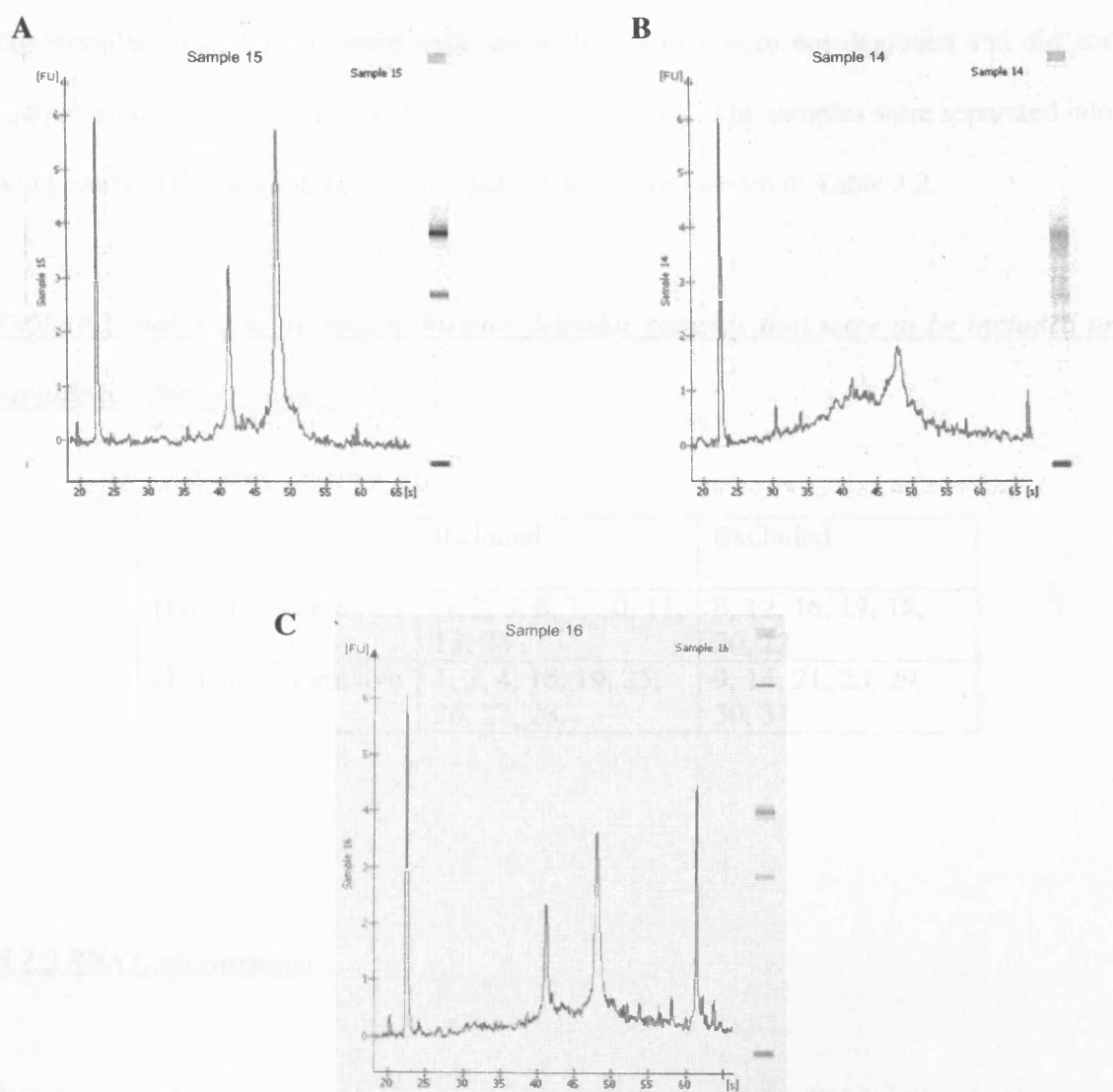


Figure 3.18 Electropherograms showing examples of: *A, a non-degraded sample with no contamination; B, a degraded sample; and C, a contaminated sample of human detrusor RNA.*

The samples to be used in gene expression studies that were not degraded and did not contain impurities were samples are shown in Table 3.2. The samples were separated into two groups, stable and overactive human detrusor, also shown in Table 3.2.

Table 3.2 Stable and overactive human detrusor samples that were to be included or excluded in further studies

	Included	Excluded
Human – stable	A, 2, 5, 6, 7, 10, 11, 13, 24	8, 12, 16, 17, 18, 20, 22
Human - overactive	1, 3, 4, 15, 19, 25, 26, 27, 28,	9, 14, 21, 23, 29, 30, 31

3.2.2 RNA Concentration

For the samples to be used in gene expression analysis the RNA concentration was determined using the Genequant calculator spectrophotometer. The equipment had an internal calibration which enabled the sample concentrations to be determined. The absorbance at 260 and 280 nm was measured. The purity of the samples was measured by the Genequant as the A260/A280 ratio. A ratio of approximately two indicates good purity (Genequant website). The concentrations and ratios of the samples are shown in Table 3.3. The ratios obtained are close to the preferred value of two implying the samples are pure which concurs with the gel images and electropherogram results.

Table 3.3. RNA sample concentration and A260/A280 ratio

Sample group	Sample	Concentration $\mu\text{g}/\mu\text{l}$	260/280 ratio
Stable	A	0.028	1.84
	2	0.107	2
	5	0.087	1.92
	6	0.107	1.95
	7	0.016	1.97
	10	0.038	1.96
	11	0.064	1.96
	13	0.067	2.11
	24	0.033	1.94
Overactive	1	0.063	1.98
	3	0.026	2.133
	4	0.050	1.88
	15	0.052	2.10
	19	0.137	2.10
	25	0.091	2.16
	26	0.079	1.97
	27	0.061	1.92
	28	0.062	1.95

3.3 RELATIVE EXPRESSION OF ENTPDASE 1, 2, 3, AND 5 GENES

After determination of the RNA concentration of each of the human detrusor samples they were reverse transcribed to cDNA. This enabled TaqMan RT-PCR to be performed on each of the samples to investigate the expression of ENTPDase 1, 2, 3, and 5 genes in human bladder. E-NTPDase 1, 2 and 3 were investigated because they are surface bound enzymes that could degrade nerve-mediated released ATP in the neuromuscular junction and may therefore influence contractile function. E-NTPDase 5 was selected from the intracellular membrane bound ectonucleotidases as a control to compare relative expression levels and any differences between the two sample groups.

In the TaqMan RT-PCR as amplification of the gene of interest and the endogenous control continues with each cycle with a threshold cycle (C_T) is reached. This is the time-point expressed in the number of PCR cycles, at which the fluorescence signal generated by probe cleavage reaches a detectable threshold. FAM was the reporter dye for the gene of interest probe and VIC for the 18s endogenous control. The lower the C_T number the greater the amount of gene present in the original detrusor sample.

3.3.1 ENTPDase 1

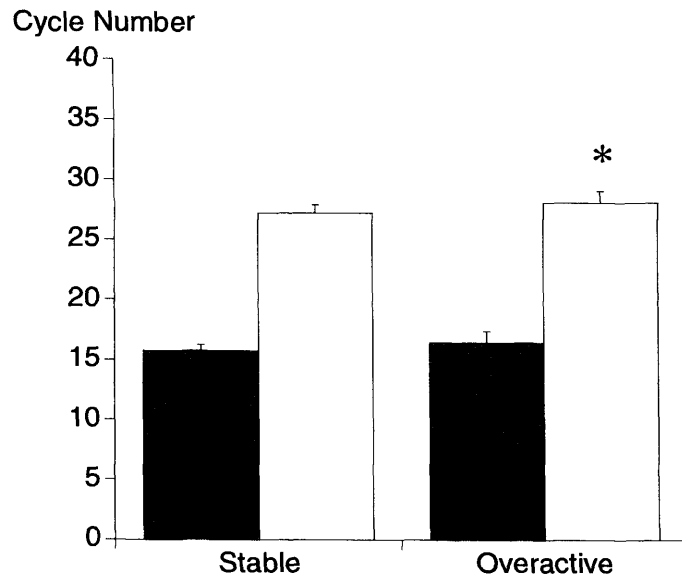
Each of the samples was prepared and measured in triplicate, the C_T numbers for the triplicates were averaged to give a C_T number for ENTPDase 1 gene in each sample. The

18s control C_T average of all the samples taken as the average of the triplicate averages, was 16.02 ± 0.73 and the C_T average for ENTPDase 1 was 27.67 ± 0.88 .

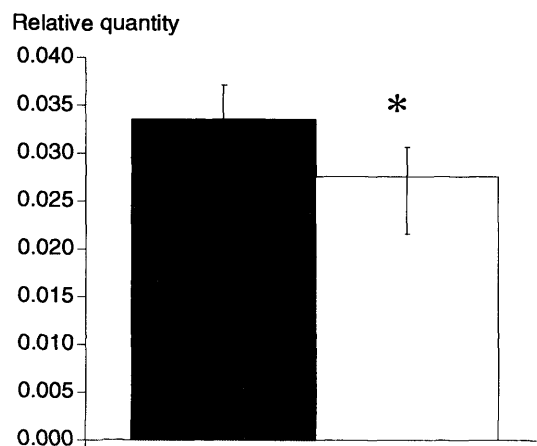
The detrusor samples were then separated into two groups, stable and overactive, to determine if there was any difference in the expression of ENTPDase 1 gene between them (Figure 3.19). The average C_T numbers for the 18s control were not different between the two groups (values). However, there was a significant difference between the two groups in the expression of ENTPDase 1 gene. Average C_T for the stable samples was 27.22 ± 0.5 and the overactive group was 28.1 ± 1.0 , indicating reduced RNA levels in the overactive group.

Further analysis of the data was carried out to incorporate the internal control data in the results. The proportion of transcript of the gene of interest present compared to that of control (18s) was determined for each sample. A comparison of the medians (25th and 75th quartiles) of the relative quantity of ENTPDase 1 between stable and overactive detrusor is shown in Figure 3.20. The median relative quantity of ENTPDase 1 in stable detrusor was 0.0336 (quartiles 0.0291 and 0.0371) compared to 0.0276 (quartiles 0.0216 and 0.0307) in overactive detrusor. When compared, using a Mann Whitney U-rank test, the relative quantity of ENTPDase 1 was significantly less in the overactive detrusor samples compared to the stable samples.

These results suggest that there is less ENTPDase 1 cDNA present in overactive detrusor samples compared to samples from stable bladders.



*Figure 3.19 Comparison of threshold cycle number for VIC labelled control gene (18s) (grey) and FAM labelled ENTPDase 1 gene (white) between stable and overactive detrusor samples. Mean \pm standard deviation, * $p < 0.05$ paired Student's t- test*



*Figure 3.20 Comparison of relative quantity of ENTPDase 1 gene compared to control gene in stable (grey) and overactive (white) detrusor samples. Median, 25th and 75th quartiles. * $p > 0.05$ Mann Whitney U-test.*

3.3.2 ENTPDase 3

The triplicate threshold cycle numbers obtained from the ENTPDase 3 assay were analysed in the same way as ENTPDase 1. The control gene C_T value was 15.4 ± 0.8 . The average C_T value for ENTPDase 3 in all the detrusor samples was 33.8 ± 1.9 .

When the samples were separated into the groups of stable and overactive, Figure 3.21, there was no significant difference between either the expression of control or ENTPDase 3. The C_T values for stable and overactive samples were 33.8 ± 1.5 and 34.5 ± 2.1 respectively.

However when the proportion of ENTPDase 3 present compared to that of the 18s control was determined there was a difference between the stable and overactive groups. The relative median quantity for stable, 0.000362 (quartiles 0.000227 and 0.00061), was significantly greater compared to overactive, 0.00204 (quartiles 0.000311 and 0.000226), as shown in Figure 3.22.

When the internal control of each sample was taken into account in the analysis of ENTPDase 3, the data showed that there was less ENTPDase 3 cDNA present in overactive detrusor samples compared to those which were from stable bladders. However, the relative quantity of ENTPDase 3 compared to ENTPDase 1 was only about 1% and so represented a minor component of overall ENTPDase expression.

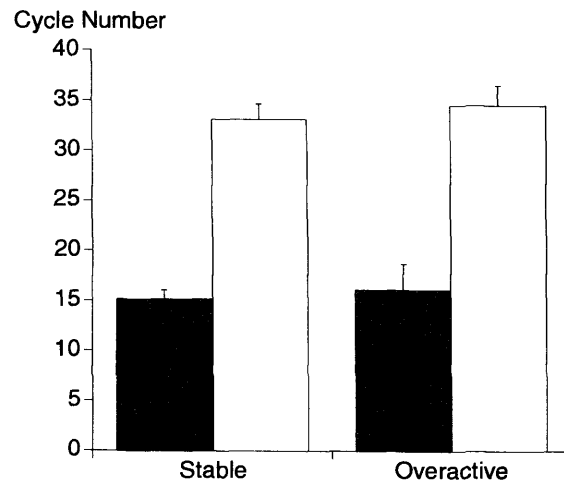


Figure 3.21 Comparison of threshold cycle number for VIC labelled control gene (18s) (grey) and FAM labelled ENTPDase 3 gene (white) between stable and over active detrusor samples. Mean \pm standard deviation.

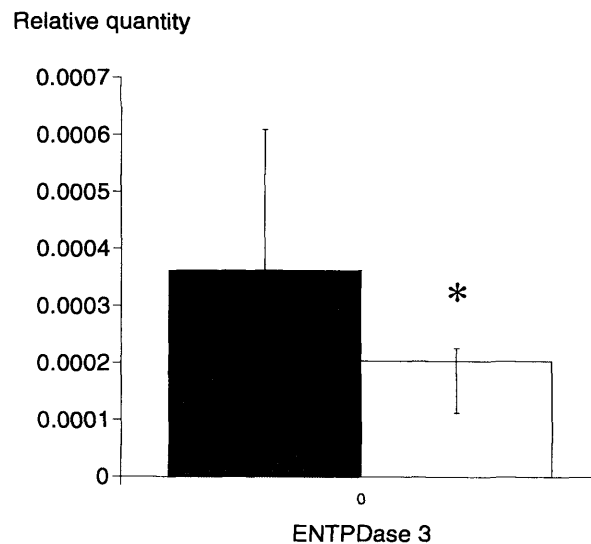


Figure 3.22 Comparison of relative quantity of ENTPDase 3 gene compared to control gene in stable (grey) and overactive (white) detrusor samples. Median, 25th and 75th quartiles. * $p < 0.05$ Mann Whitney - test.

3.3.3 ENTPDase 2

The triplicate threshold cycle numbers obtained from the ENTPDase 2 assay were analysed in the same way as ENTPDase 1. The average control C_T value was 16.9 ± 0.8 . The average C_T value for ENTPDase 2 in all the detrusor samples was 34.7 ± 1.6 indicating that ENTPDase 2 was present in the detrusor samples.

When the samples were separated into the groups of stable and overactive, Figure 3.23 panel A, there was no significant difference between the either the expression of control or ENTPDase 2. The C_T value for stable was 34.2 ± 1.1 and was 35.1 ± 1.9 for the overactive samples.

There was also no significant difference in the median relative quantity of ENTPDase 2 between stable and overactive detrusor samples (Figure 3.23, panel B). Stable detrusor samples had a median relative quantity of 0.000417 (quartiles 0.000239 and 0.000808) and overactive detrusor samples was 0.000512 (quartiles 0.000304 and 0.000705).

These results suggest that there was no difference in the amount of ENTPDase 2 gene present in stable and overactive detrusor samples.

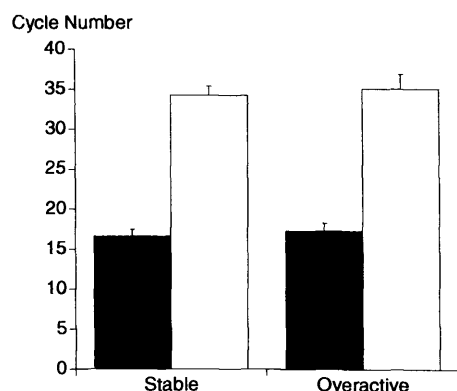
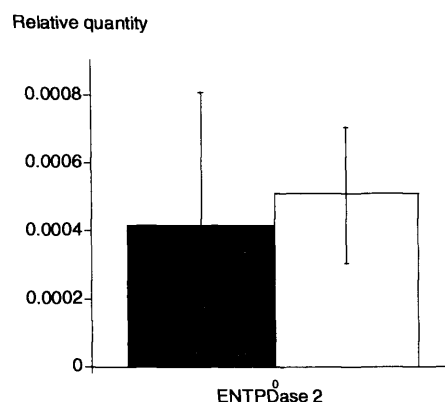
A**B**

Figure 3.23 Comparison of expression of ENTPDase 2 between stable and overactive detrusor samples. A, comparison of threshold cycle number for VIC labelled control gene (grey) and FAM labelled ENTPDase 2 (white), mean \pm standard deviation. B, comparison of relative quantity, stable (grey), overactive (white), median and 25th and 75th quartiles.

3.3.4 ENTPDase 5

The average cycle threshold number for the internal control gene in all the detrusor samples in the ENTPDase 5 assay was 19.1 ± 3.2 . The C_T value for the ENTPDase gene was 33.8 ± 1.0 thus indicating the presence of ENTPDase 5 in the detrusor samples.

When the detrusor samples were separated into stable and over-active groups there was no significant difference in the mean control and ENTPDase 5 gene C_T values between

the two groups as shown in Figure 3.24 panel A. The mean control C_T values for the stable and overactive groups were 19.4 ± 3.4 and 18.8 ± 3.2 respectively and 33.7 ± 0.7 and 33.9 ± 1.2 for the ENTPDase 5 gene.

When the relative quantity of ENTPDase 5 compared to control was compared between the two groups, Figure 3.24 panel B, there was no significant difference. The median relative quantity of ENTPDase 5 in stable detrusor was 0.00545 (quartiles 0.000796 and 0.03873) and 0.003123 (quartiles 0.000486 and 0.005828) in overactive detrusor samples.

The results show that there was no significant difference in the amount of ENTPDase 5 in stable detrusor compared with over-active human detrusor.

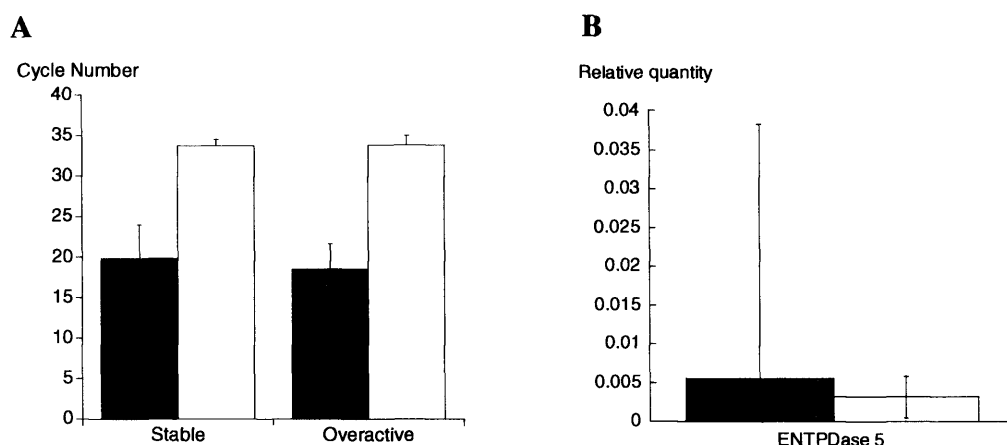


Figure 3.24 Comparison of expression of ENTPDase 5 between stable and overactive detrusor samples. A, comparison of threshold cycle number for VIC labelled control gene (grey) and FAM labelled ENTPDase 5 (white), mean \pm standard deviation. B, comparison of relative quantity, stable (grey), overactive (white), median and 25th and 75th quartiles.

3.3.5 Relative quantities of ENTPDase 1, 2 and 3 expression

When comparing the relative quantity of E-NTPDase 1, 2, and 3 gene expression compared to the control gene, E-NTPDase 1 was much greater in both stable and overactive human detrusor samples, Figure 3.25.

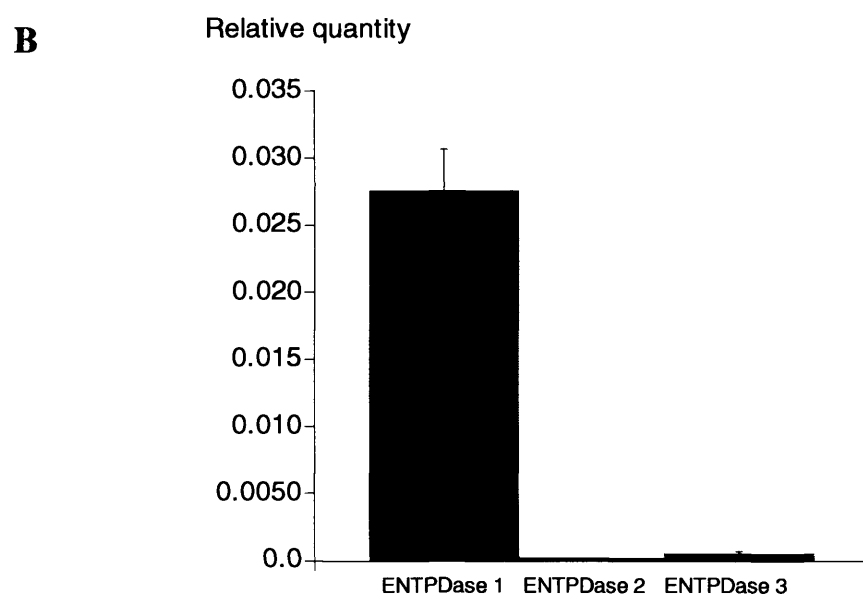
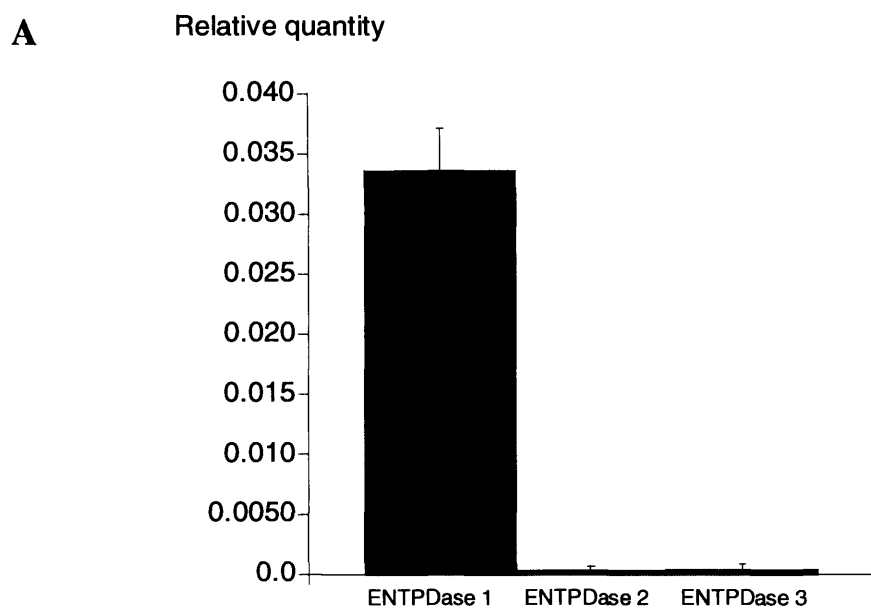


Figure 3.25 Comparison of relative quantity of E-NTPDase 1, 2, and 3 genes compared to control gene in A) stable and B) overactive human detrusor samples. median and 25th and 75th quartiles.

The results show that all four ectonucleotidase are present in detrusor from human bladders although E-NTPDase 1 expression was significantly greater than E-NTPDase 2, 3 and 5. Expression of E-NTPDase 1 and 3 was significantly greater in detrusor samples from stable bladders compared to those from overactive bladders. Some overactive bladders have an ATP component to nerve-mediated contraction which could be a result of decreased breakdown of ATP as a consequence of reduced ectonucleotidase expression particularly as the highly expressed E-NTPDase 1 is reduced in detrusor samples from overactive bladders.

3.4 ATP DEGRADATION RATE

3.4.1 Guinea pig detrusor

The rate of degradation of five concentrations of ATP by guinea pig detrusor was measured over a period of 30 minutes to investigate the activity of ectonucleotidases. The measurements of the ATP concentration in the solution surrounding a detrusor strip were taken at time points of 0, 5, 10, 20 and 30 minutes. Light units measured by the luminometer were converted to ATP concentrations using the ATP calibration curve. Figure 3.26 shows an example of the degradation of the five ATP concentrations over the thirty minutes. The initial concentrations of ATP plotted in Figure 3.26 and then used to determine degradation rates were those measured in the time zero aliquots; ie immediately following addition of stock ATP and mixing of the solution containing the detrusor sample. The breakdown of ATP followed an approximately linear pattern from which the rate of degradation at each concentration was determined. Detrusor samples were weighed and therefore rates for each of the detrusor samples could be normalised. The calculated rates in this experiment for each concentration are plotted in Figure 3.27 for total ATPase activity to determine the maximum rate of breakdown (V_{\max}) and the ATP concentration required to produce half the maximum rate (K_m).

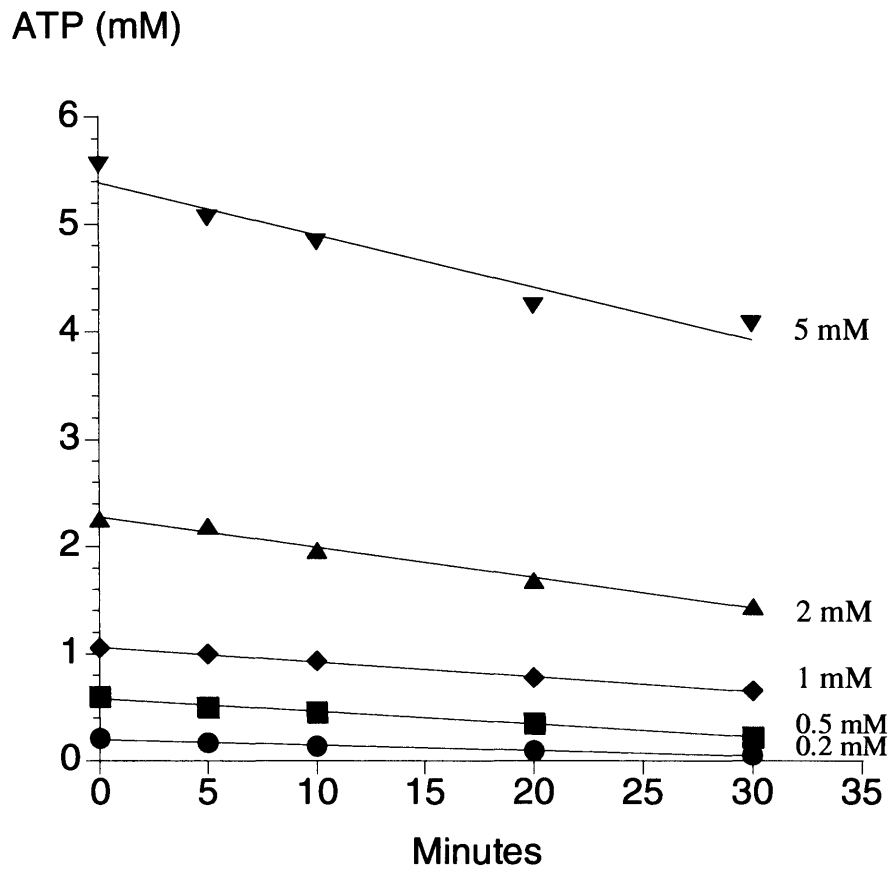


Figure 3.26 Example of ATP degradation over time by guinea pig detrusor. Initial concentrations of ATP were 0.2, 0.5, 1, 2, and 5 mM.

ATP degradation rate
(nmol.s⁻¹.mg⁻¹)

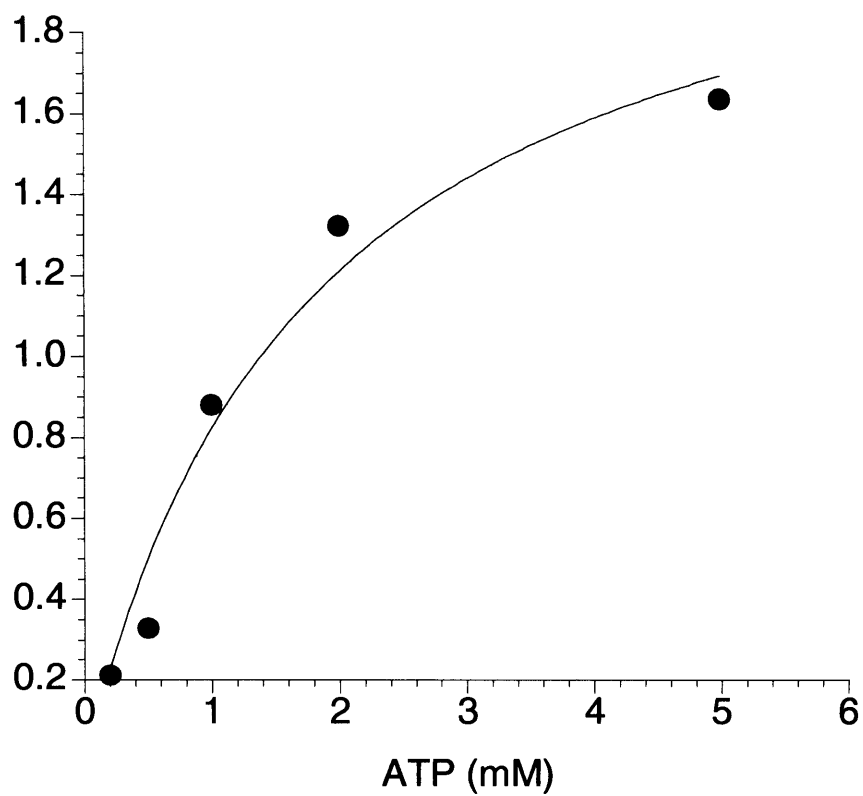


Figure 3.27 Plot of ATP degradation rates at varying ATP concentrations for the experiment illustrated in figure 3.26.

In eight guinea pig preparations the average ATP degradation rates at each of the five ATP concentrations were plotted as shown in figure 3.28. The data from individual experiments give an average estimated V_{\max} of $3.7 \pm 2.3 \text{ nmol.s}^{-1}.\text{mg}^{-1}$ and a K_m of $4.32 \pm 2.41 \text{ mM}$ for the total ATPase enzyme activity in guinea pig detrusor.

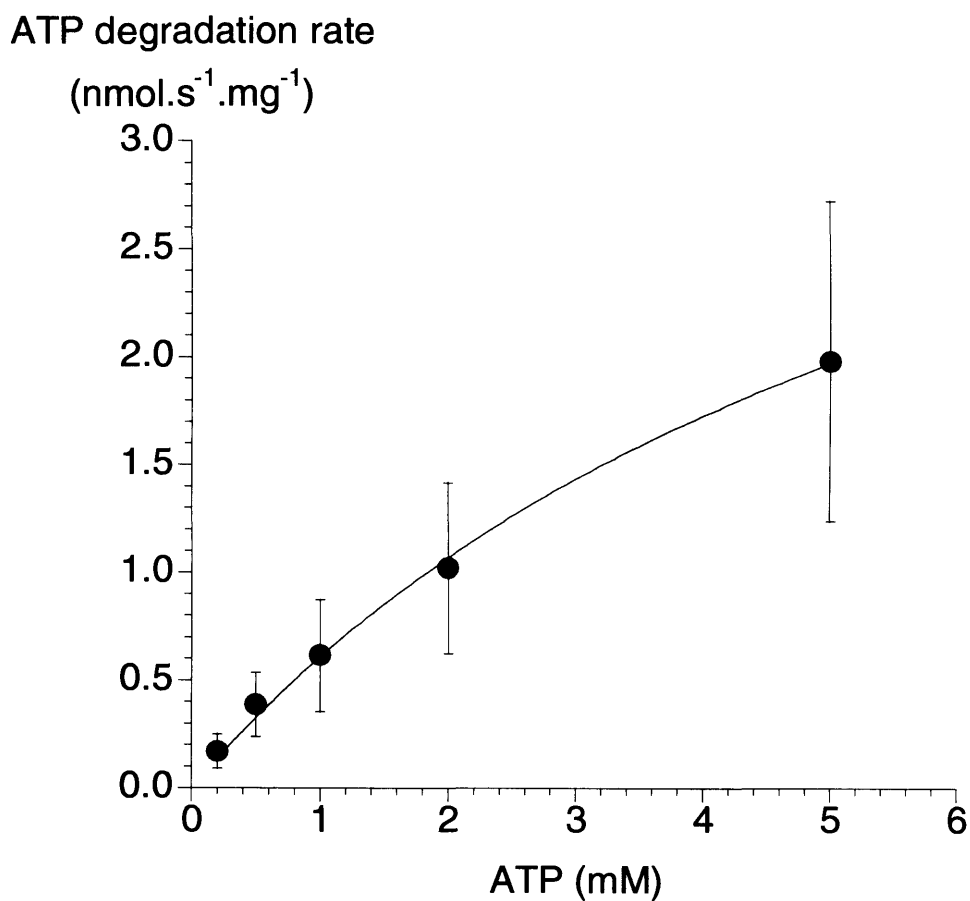


Figure 3.28 Average ATP degradation rate in guinea pig detrusor. Mean \pm standard deviation.

In order to determine the ectonucleotidase-dependent rate of ATP degradation the inhibitor ARL 67156 was used. Figure 3.29 shows an example of the rates of degradation achieved at 1 and 5 mM ATP in the presence and absence of 100 μ M ARL 67156. ARL 67156 caused the ATP breakdown rate in the example to be reduced by 11.7% and 47.3% for 1 and 5 mM respectively in this example. The ARL 67156- sensitive fraction of the ATP breakdown rate was taken as the ectonucleotidase rate.

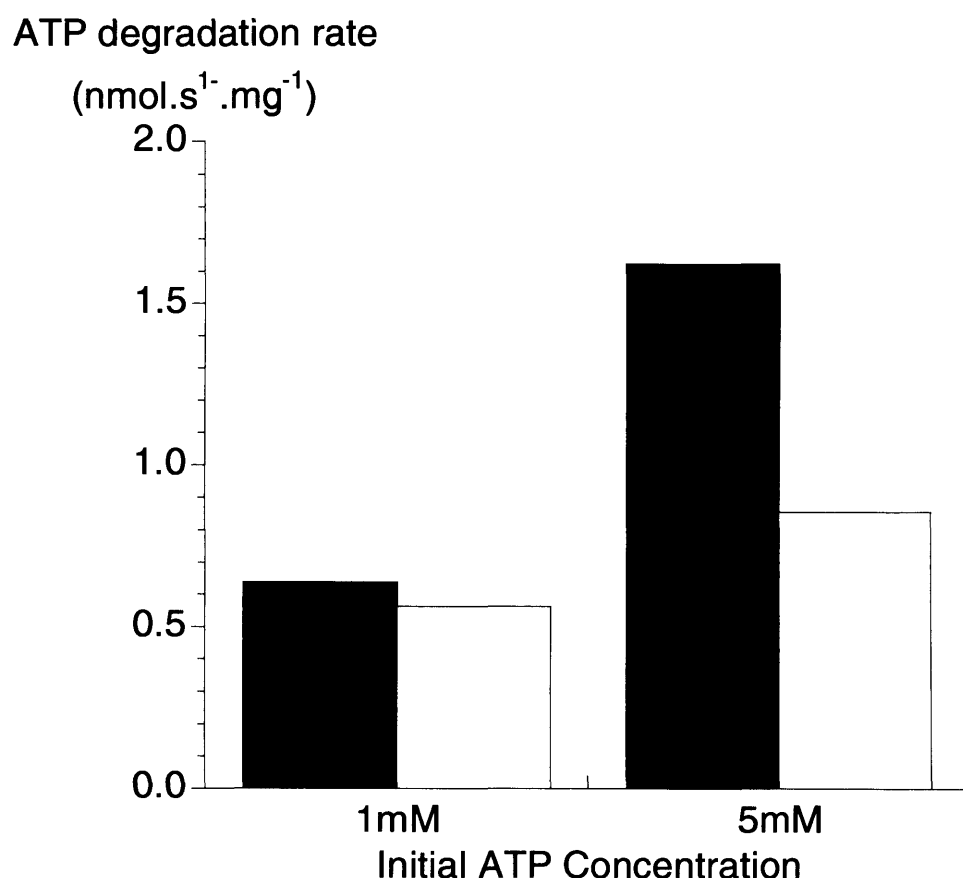
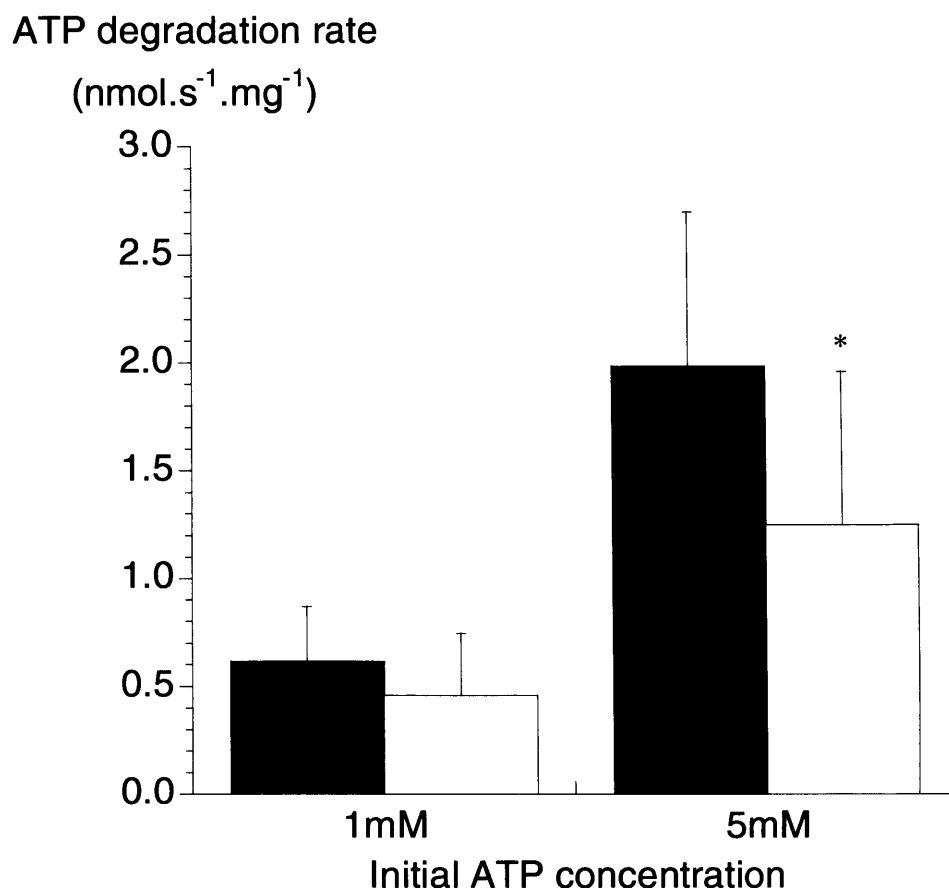


Figure 3.29 Example of ATP degradation rates at 1 and 5 mM ATP in the absence and presence of 100 μ M ARL 67156. Control, black; ARL 67156, white.

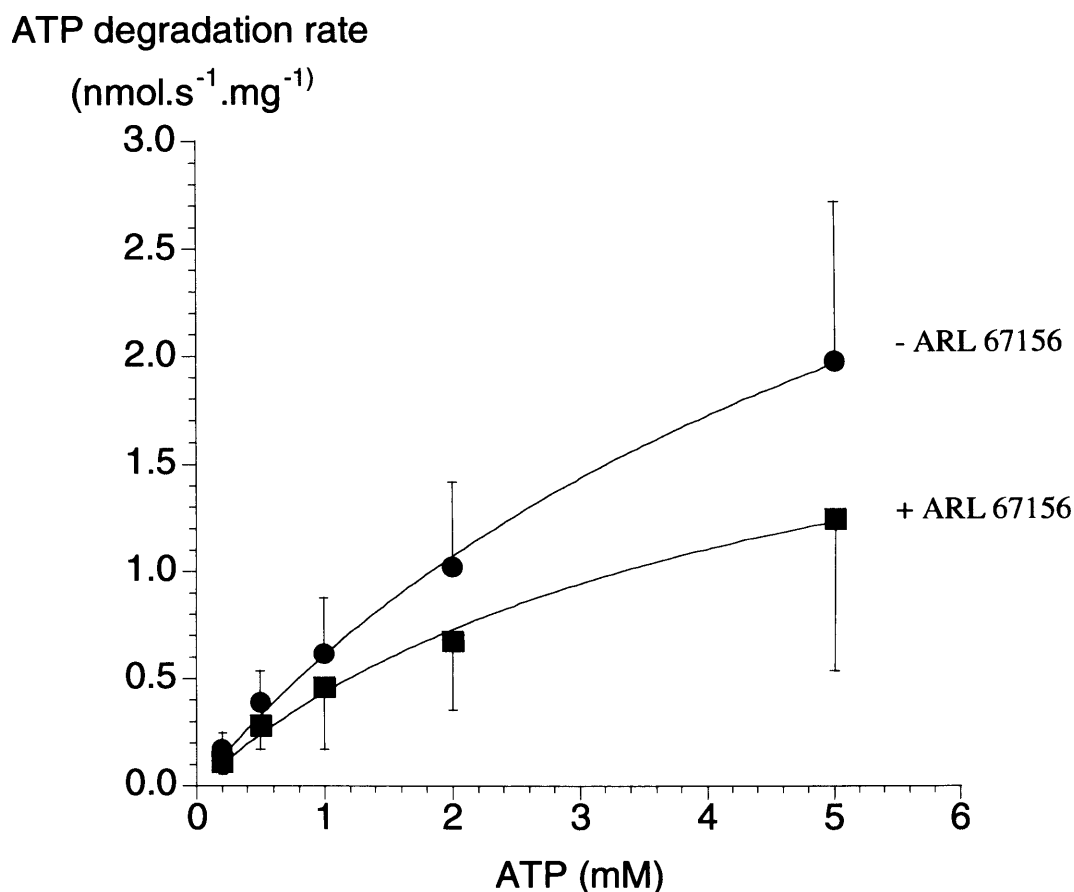
In the presence of the ectonucleotidase inhibitor ARL 67156 the average breakdown of ATP in the eight guinea pig detrusor preparations was reduced by $19.6 \pm 28.8 \%$ with 1 mM ATP and by $39.1 \pm 29.0 \%$ with 5mM ATP (Figure 3.30). In the latter case the reduction of activity was significant. A paired Student's t-test was used to compare the rates of degradation of ATP in the presence and absence of ARL 67156. When the initial concentration of ATP was 5mM the ectonucleotidase sensitive component rate of breakdown was $0.62 \pm 0.54 \text{ nmol.s}^{-1}.\text{mg}^{-1}$.



*Figure 3.30 guinea pig - average ATP degradation rate at 1 and 5 mM ATP in the presence of ARL 67156. Control: black, ARL 67156: white. Mean \pm standard deviation, * $p < 0.05$ paired Student's t-test.*

An alternative formulation of the ARL-67156-sensitive component was to calculate the V_{\max} value from the rate vs [ATP] plot in the absence and presence of the inhibitor. The difference between the two V_{\max} values was the desired variable. Figure 3.31 shows the average breakdown rates in the absence and presence of 100 μ M ARL 67156 over the

full range of ATP concentrations. The estimated V_{\max} was significantly reduced from 3.7 ± 2.3 to 2.2 ± 1.3 $\text{nmol.s}^{-1}.\text{mg}^{-1}$. However, the K_m was unaltered; 4.32 ± 2.41 vs 3.58 ± 2.33 μM , respectively.



*Figure 3.31 Average ATP degradation rates at varying ATP concentrations in the presence (squares) and absence (circles) of ARL 67156. Mean \pm standard deviation, * $p < 0.05$ paired Student's t -test.*

To ascertain the rates of the ectonucleotidase activity only, the rates obtained at each ATP concentration in the presence of $100 \mu\text{M}$ ARL 67156 were subtracted from those in

control conditions. The subtracted rates were plotted (Figure 3.32) to yield an average V_{\max} of $1.1 \pm 1.4 \text{ nmol.s}^{-1}.\text{mg}^{-1}$ and a K_m of $1.47 \pm 0.99 \text{ mM}$ for ectonucleotidase activity in guinea pig detrusor.

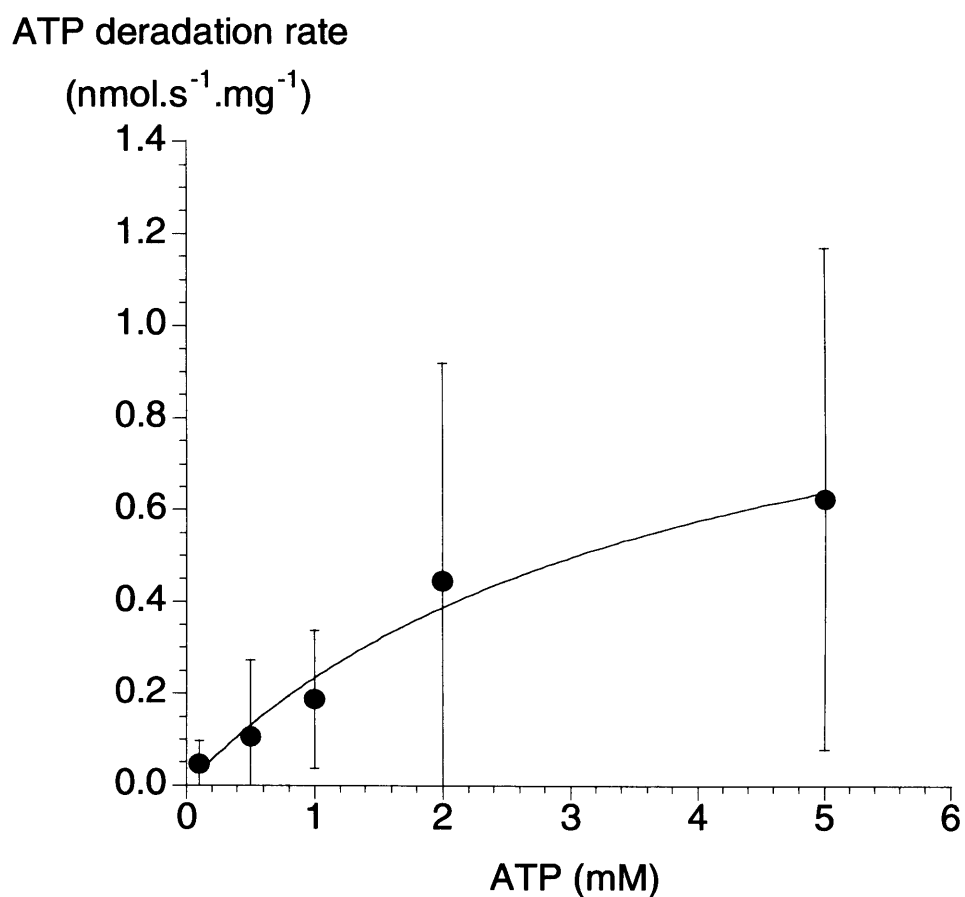


Figure 3.32 ARL 67156 sensitive average ATP degradation rates at varying ATP concentrations Mean \pm standard deviation

Figure 3.33 shows each of the components of ATP breakdown, total ATPase, ARL 67156 resistant ATPase and ARL 67156 sensitive ATPase , ectonucleotidase, activity over the full range of ATP concentrations tested.

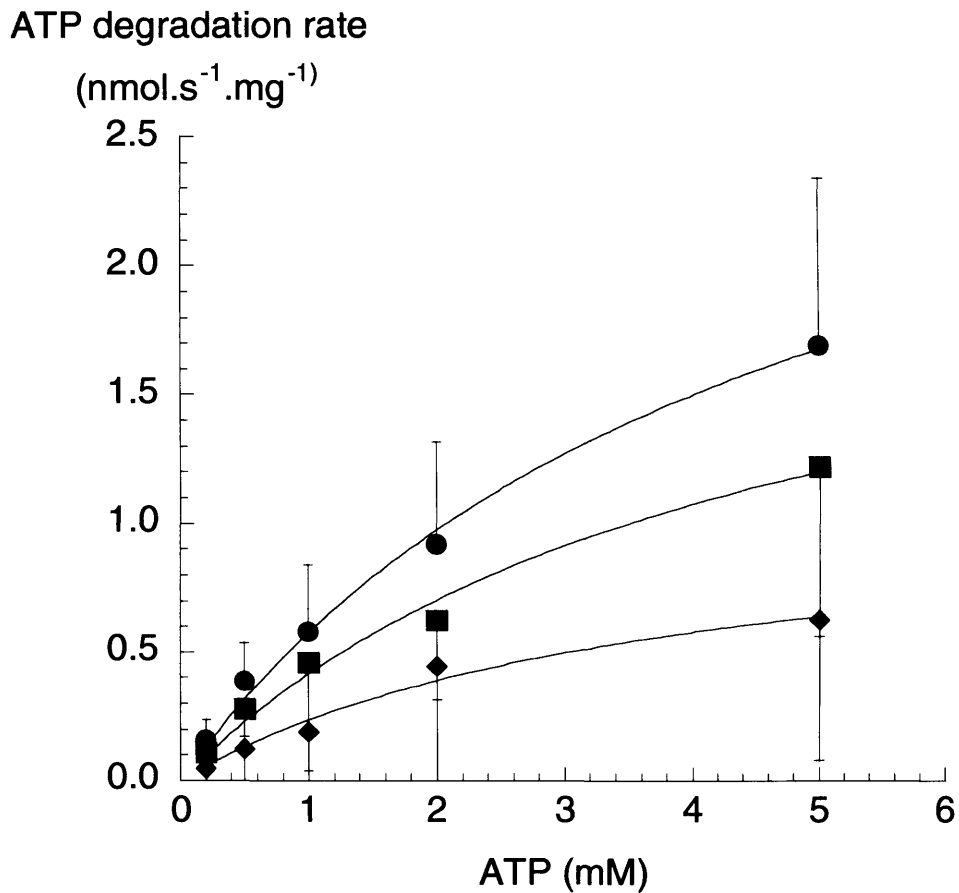


Figure 3.33 ATP degradation over a range of ATP concentrations by guinea pig detrusor. Control: circles, ARL 67156 insensitive: squares, ARL 67156 sensitive: diamonds. Mean \pm standard deviation.

3.4.2 Human detrusor – stable bladders

Ectonucleotidase activity was also measured in sample pieces of human detrusor from stable bladders. As with guinea-pig samples, the degradation rates with five initial concentrations of ATP (0.2, 0.5, 1, 2, and 5 mM) were measured over 30 minutes. Figure 3.34 shows an example where the concentration of ATP declined at all concentrations over this period. The rate of decline was greater as the initial concentration of ATP increased, as shown in the plot of Figure 3.35 for this experiment.

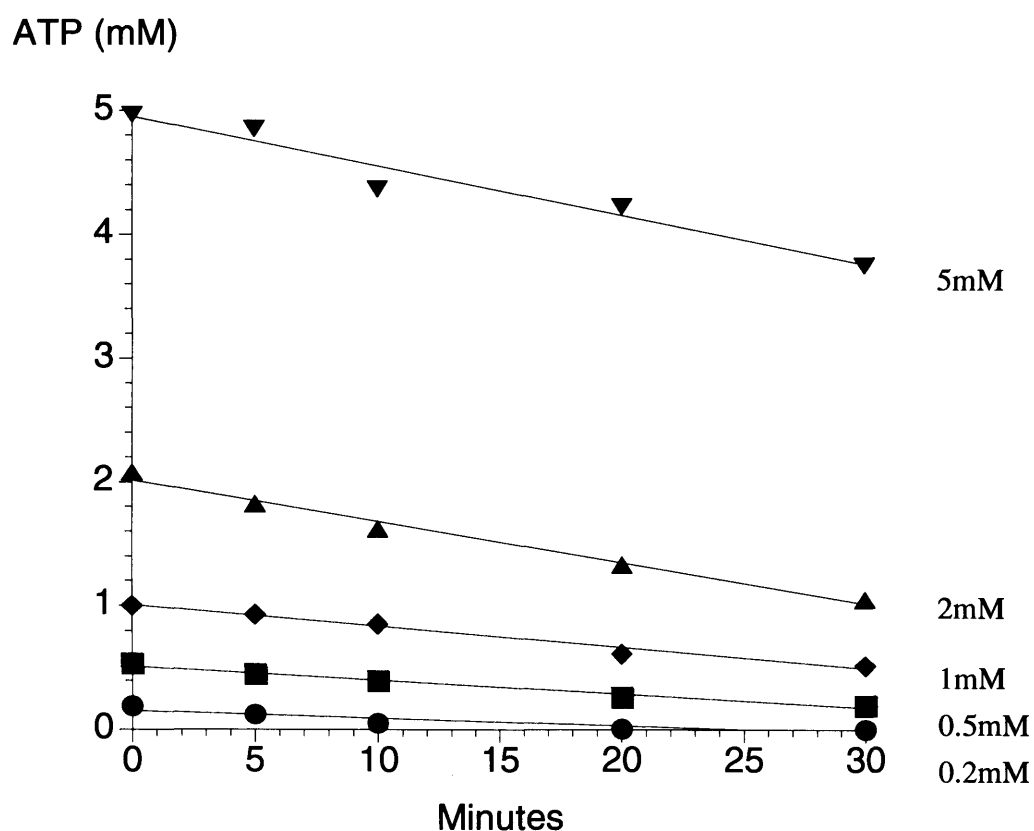


Figure 3.34 Example of ATP degradation over time by human detrusor from a stable bladder. Initial concentrations of ATP were 0.2, 0.5, 1, 2, and 5 mM.

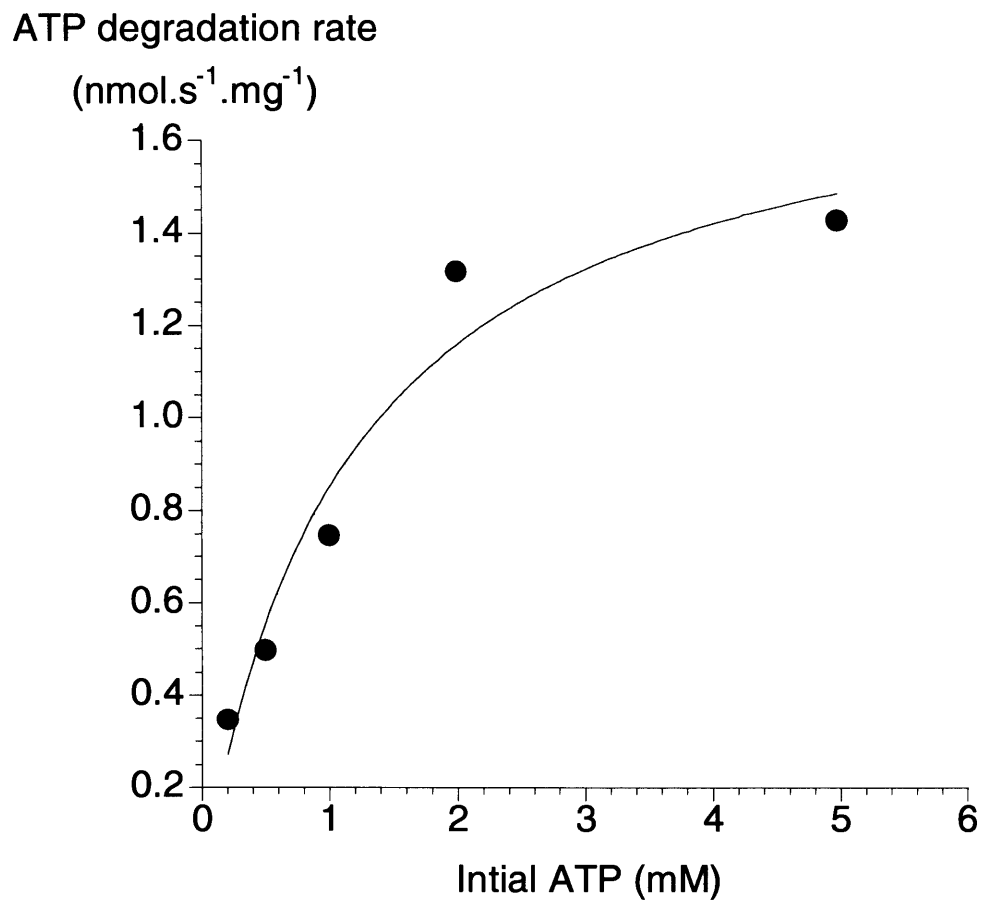


Figure 3.35 Human detrusor from a stable bladder - ATP degradation rates at varying ATP concentrations from the data shown in figure 3.34.

The average degradation rates in control conditions as a function of the initial ATP concentrations are shown in Figure 3.36. The average estimated V_{\max} for total ATPase activity in stable human detrusor (n=13) was $2.4 \pm 0.9 \text{ nmol.s}^{-1}.\text{mg}^{-1}$. The average estimated K_m was $4.47 \pm 2.48 \text{ mM}$.

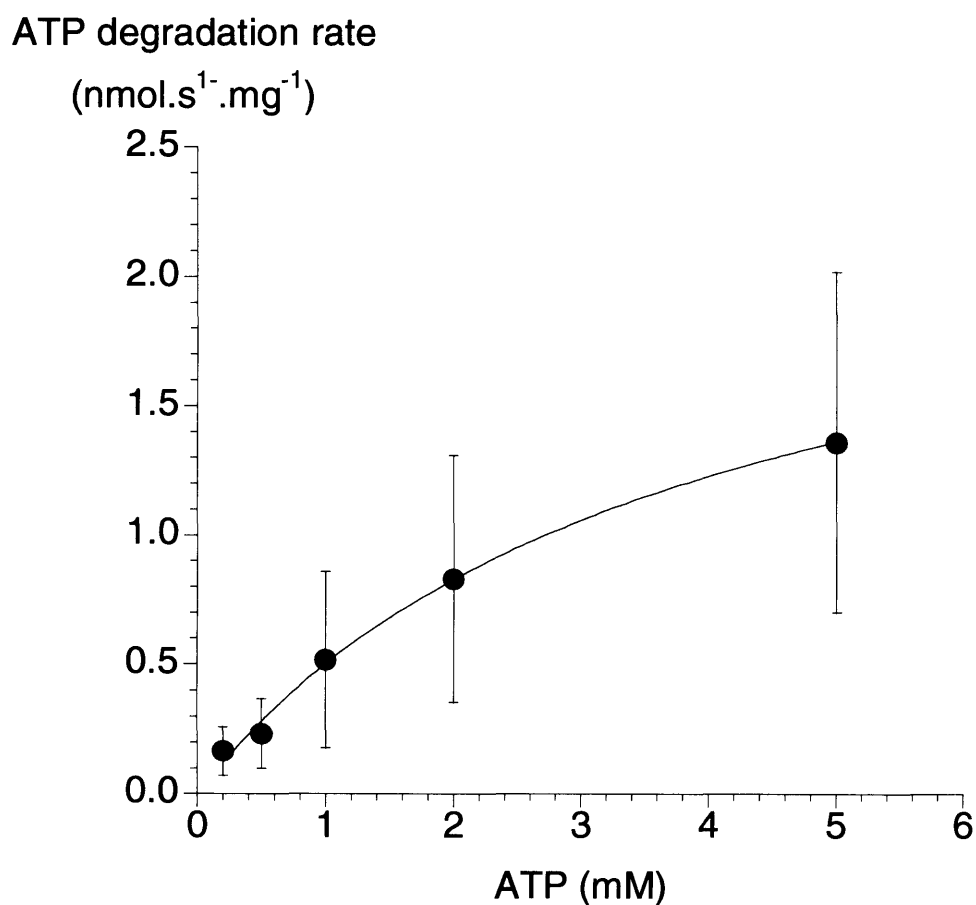
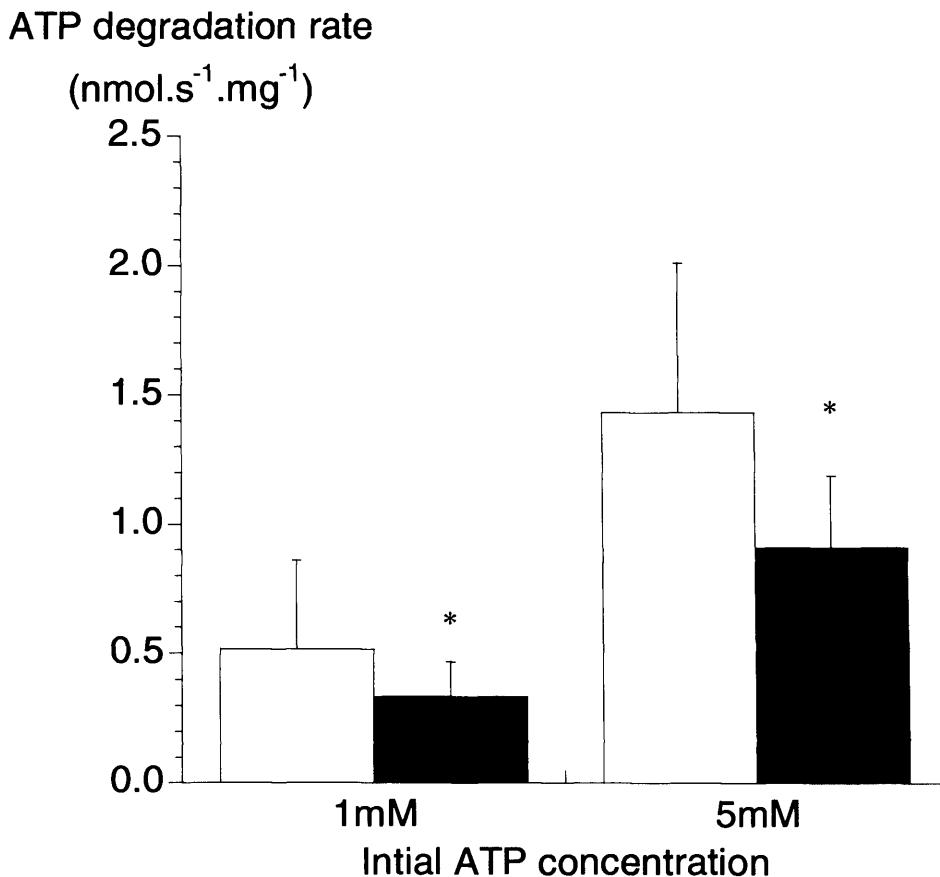


Figure 3.36 Average ATP degradation rates in human detrusor from stable bladders.

Mean \pm standard deviation

When the experiment was performed in the presence of ARL 67156 the rates of ATP breakdown at each of the five ATP concentrations were reduced significantly when compared to those in control conditions. The bar chart in Figure 3.37 illustrates this at two particular concentrations of initial ATP, 1 and 5 mM. The rate at each concentration was reduced by a similar amount: 35.5% with 1 mM ATP and 36.4% with 5 mM ATP. At 5mM the ectonucleotidase activity resulted in an ATP breakdown rate of 0.51 ± 0.43 nmol.s⁻¹.mg⁻¹.



*Figure 3.37 Human detrusor: stable bladders- mean ATP degradation rate at 1 and 5 mM ATP in the presence (black bars) and absence (white bars) of 100 μ M ARL 67156. Mean \pm standard deviation, * $p < 0.05$ paired Student's t -test.*

Assuming ARL 67156 is completely effective at inhibiting ectonucleotidase activity the breakdown of ATP observed in the presence of ARL 67156 is the result of all ATPase activity, except ectonucleotidase activity. The average rates of breakdowns over the ATP concentration range in the presence of the inhibitor are shown along with control rates in Figure 3.38. The estimated V_{\max} was significantly reduced with ARL 67156 from $2.4 \pm$

0.9 to $1.6 \pm 0.7 \text{ nmol.s}^{-1}.\text{mg}^{-1}$; the K_m values were similar in the two conditions: 4.47 ± 2.48 vs $3.46 \pm 1.74 \text{ mM}$, respectively.

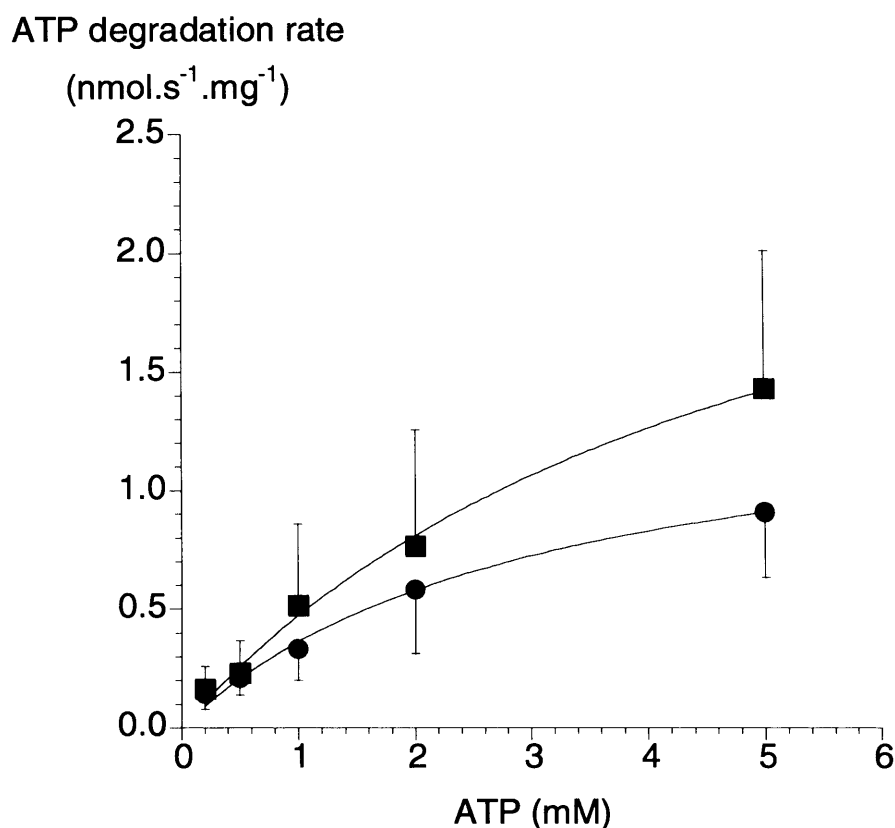


Figure 3.38 Human detrusor: stable bladders - average ATP degradation rates at varying ATP concentrations in the presence (circle) and absence (square) of 100 μM ARL 67156. Mean \pm standard deviation.

The rate of ectonucleotidase activity was determined by plotting the ARL 67156 sensitive fraction. This was calculated, as with the guinea pig samples, by subtracting the rates obtained at each ATP concentration in the presence of ARL 67156 from those obtained in control condition. The average ARL 67156 sensitive rates are plotted in

Figure 3.39. The V_{\max} estimated from the plot was $1.1 \pm 0.4 \text{ nmol.s}^{-1}.\text{mg}^{-1}$ and the K_m was $4.45 \pm 2.62 \text{ mM}$.

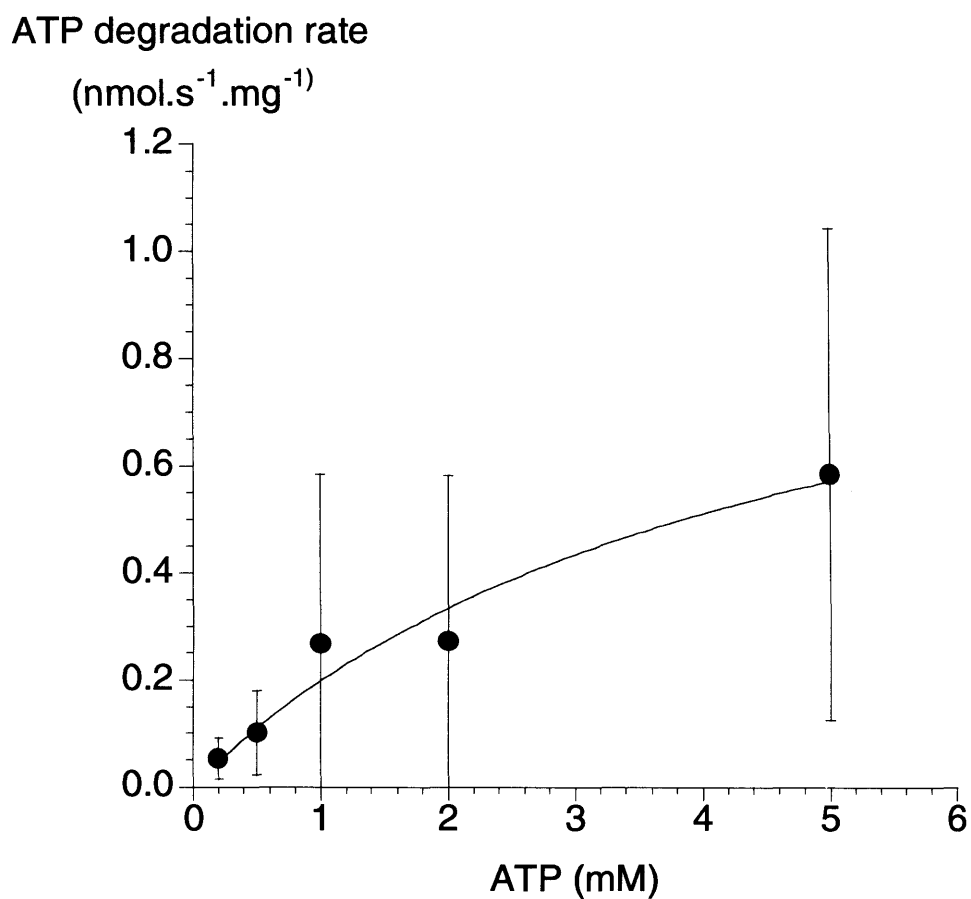


Figure 3.39 Human detrusor: stable bladders – ARL 67156 sensitive average ATP degradation rates at varying ATP concentrations Mean \pm standard deviation.

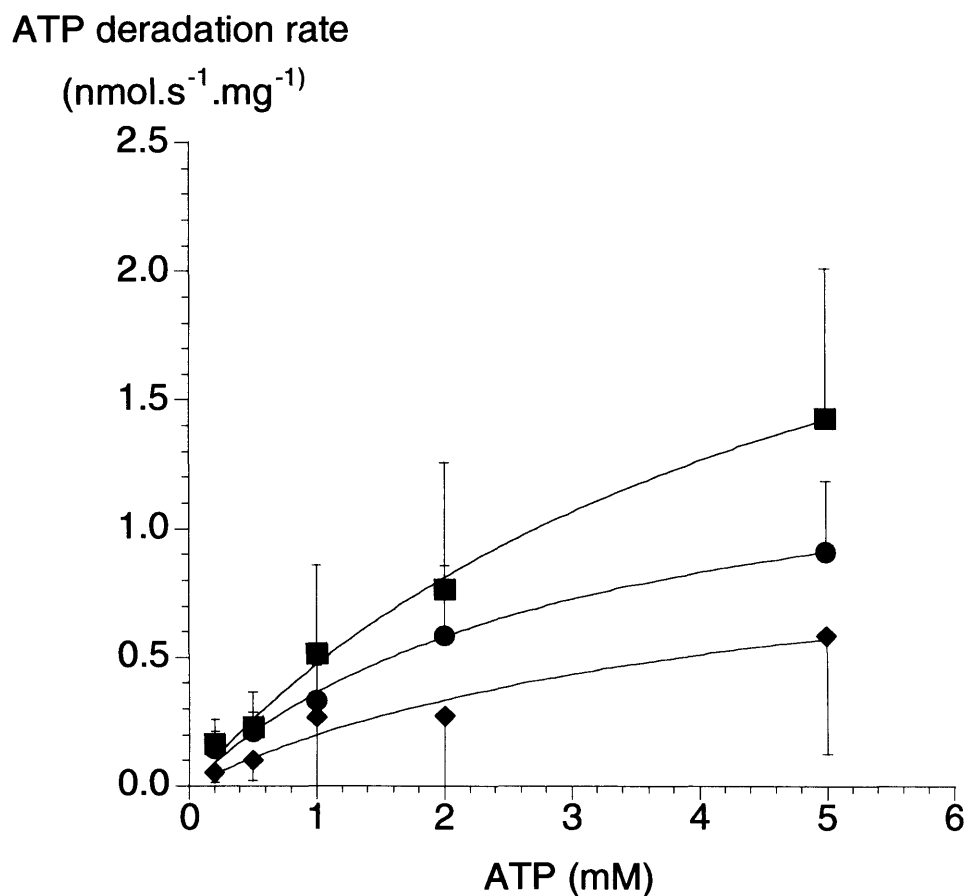


Figure 3.40 ATP degradation over a range of ATP concentrations in human detrusor from stable bladders. Control: circles, ARL 67156 insensitive: squares, ARL 67156 sensitive: diamonds. Mean \pm standard deviation.

Figure 3.40 illustrates ATP breakdown over a range of ATP concentrations by total ATPase and ARL 67156 resistant ATPase activity and ARL 67156 sensitive, taken to be ectonucleotidase activity.

3.4.3 Human detrusor – overactive bladders

The same experiments were performed on samples of detrusor from overactive human bladders (n=11). In the control experiments degradation of the five initial concentrations of ATP over the 30 minute period followed the same linear pattern of decline as with the samples from stable bladders. The amount of breakdown varied with the different initial ATP concentrations with increasing breakdown rates at greater concentrations of initial ATP. Breakdown rates were calculated for each ATP concentration for each sample. The graph in Figure 3.41 shows the average rates of breakdown of ATP in the overactive detrusor samples. The estimated V_{\max} for these samples was $2.0 \pm 1.0 \text{ nmol.s}^{-1}.\text{mg}^{-1}$ and the K_m was $3.33 \pm 1.74 \text{ mM}$.

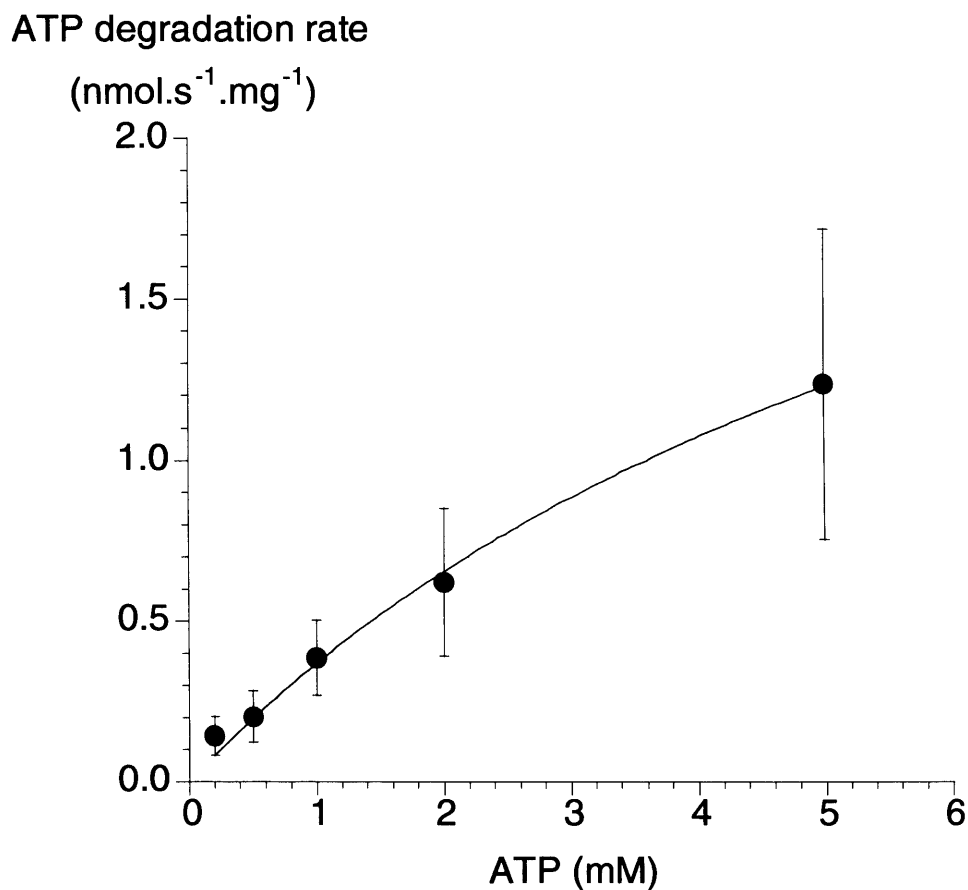


Figure 3.41 Human detrusor: stable bladders - average ATP degradation rates at varying ATP concentrations. Mean \pm standard deviation

When the experiments were carried out with the addition of ARL 67156 the degradation rates calculated were reduced when compared to control. Figure 3.42 shows that with initial concentrations of ATP of 1 and 5 mM the presence of the ectonucleotidase inhibitor the breakdown rates were significantly reduced by an average of 35.7 and 46.8% respectively. The ARL 67156 sensitive component of activity with an initial ATP concentration of 5 mM degraded ATP at a rate of 0.59 ± 0.36 nmol.s⁻¹.mg⁻¹.

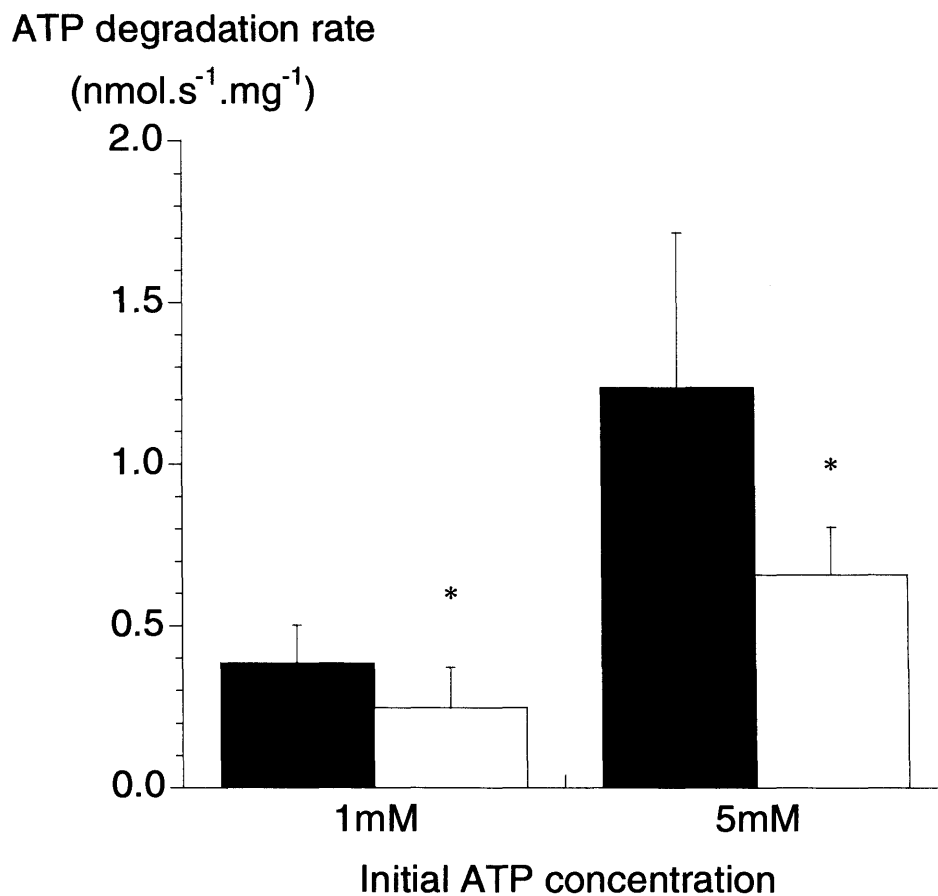


Figure 3.42 Human detrusor: overactive bladders - average ATP degradation rate at 1 and 5 mM ATP in the presence of 100 μ M ARL 67156. Control: black bars, ARL 67156: white bars. Mean \pm standard deviation, * $p < 0.05$ paired Student's t -test.

When all the average rates obtained with ARL 67156 were plotted (shown in Figure 3.43) the estimated V_{\max} of ATPase activity excluding ectonucleotidases was significantly reduced to 1.1 ± 0.4 nmol.s⁻¹.mg⁻¹ compared to control and whilst the K_m at 3.06 ± 1.43 mM was similar to control.

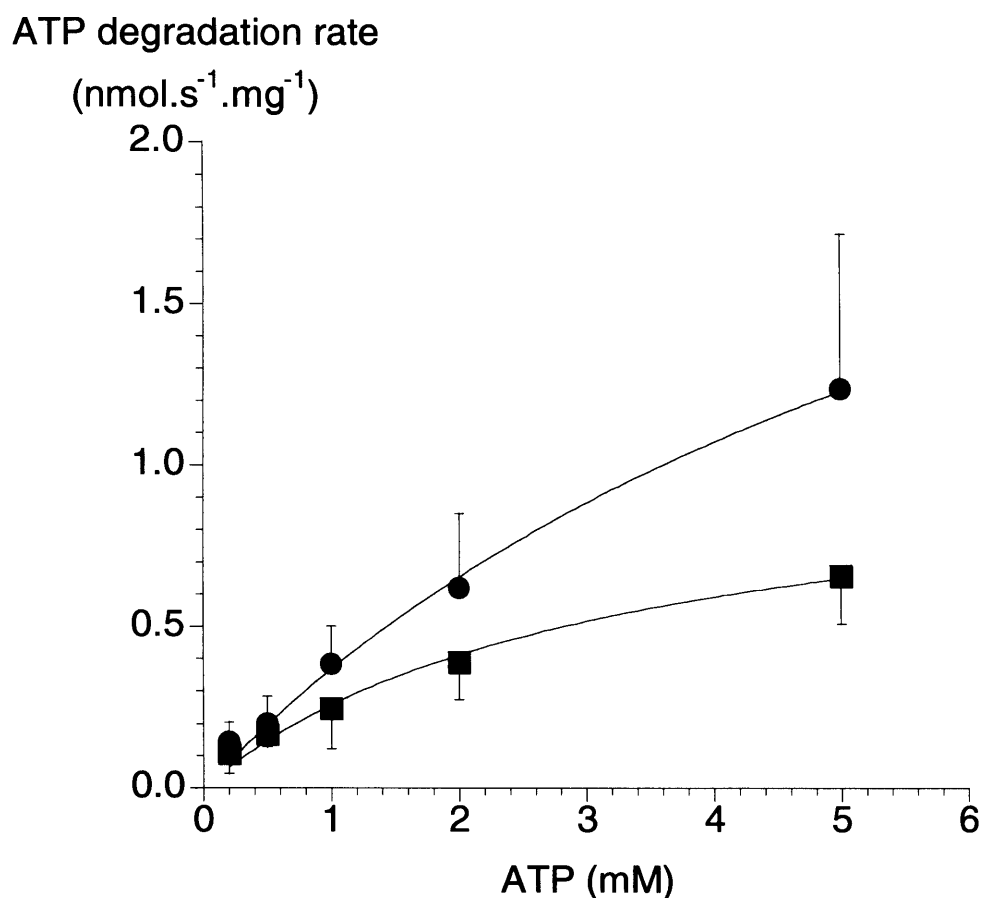


Figure 3.43 Human detrusor: overactive bladders - average ATP degradation rates at varying ATP concentrations in the presence (squares) and absence (circles) of 100 μ M ARL 67156. Mean \pm standard deviation.

In a similar way to data from human samples from stable bladders the activity of the ARL 67156 sensitive fraction was plotted (Figure 3.44). The average estimated V_{\max} , estimated from individual ARL 67156 sensitive curves was $1.2 \pm 0.9 \text{ nmol.s}^{-1}.\text{mg}^{-1}$ and was significantly smaller compared to V_{\max} of total ATPase activity. The average K_m , $3.33 \pm 2.48 \mu\text{M}$, was not different when compared that for total activity.

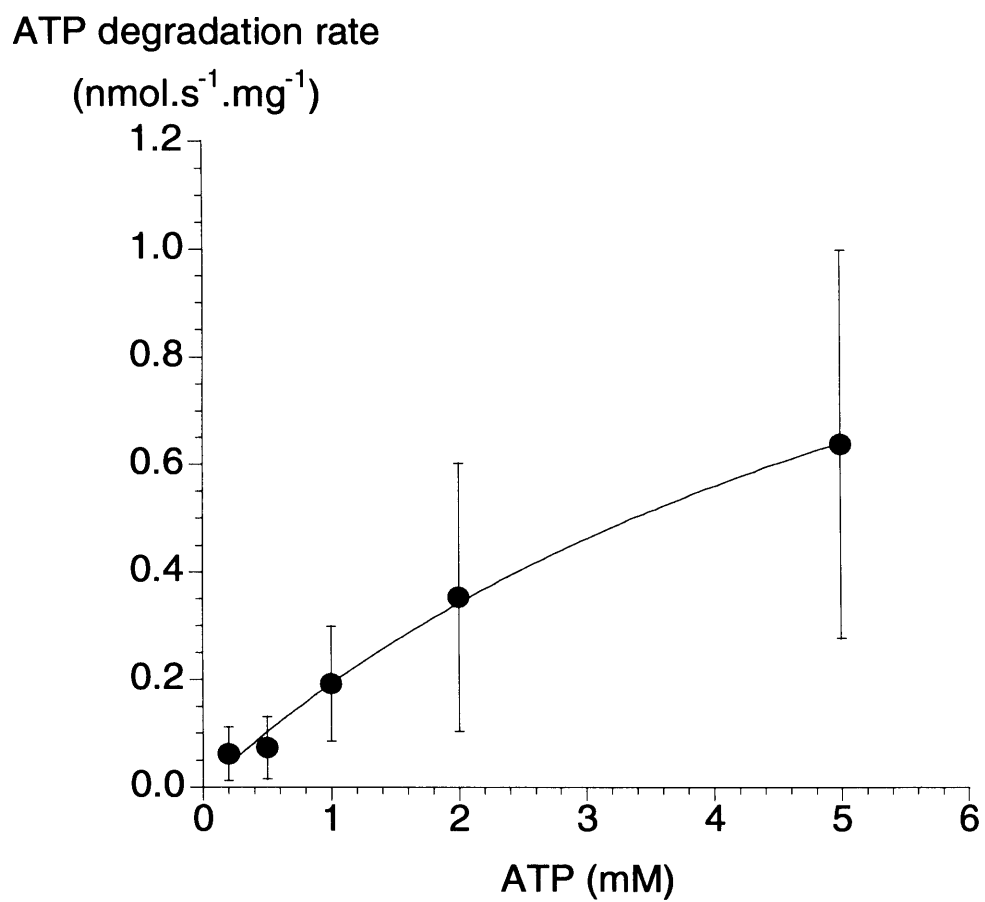


Figure 3.44. Human detrusor: overactive bladders – ARL 67156 sensitive average ATP degradation rates at varying ATP concentrations. Mean \pm standard deviation.

The components of ATP breakdown measured, total ATPase, ARL 67156 resistant ATPase and ARL 67156 sensitive ATPase activity, are shown in Figure 3.45.

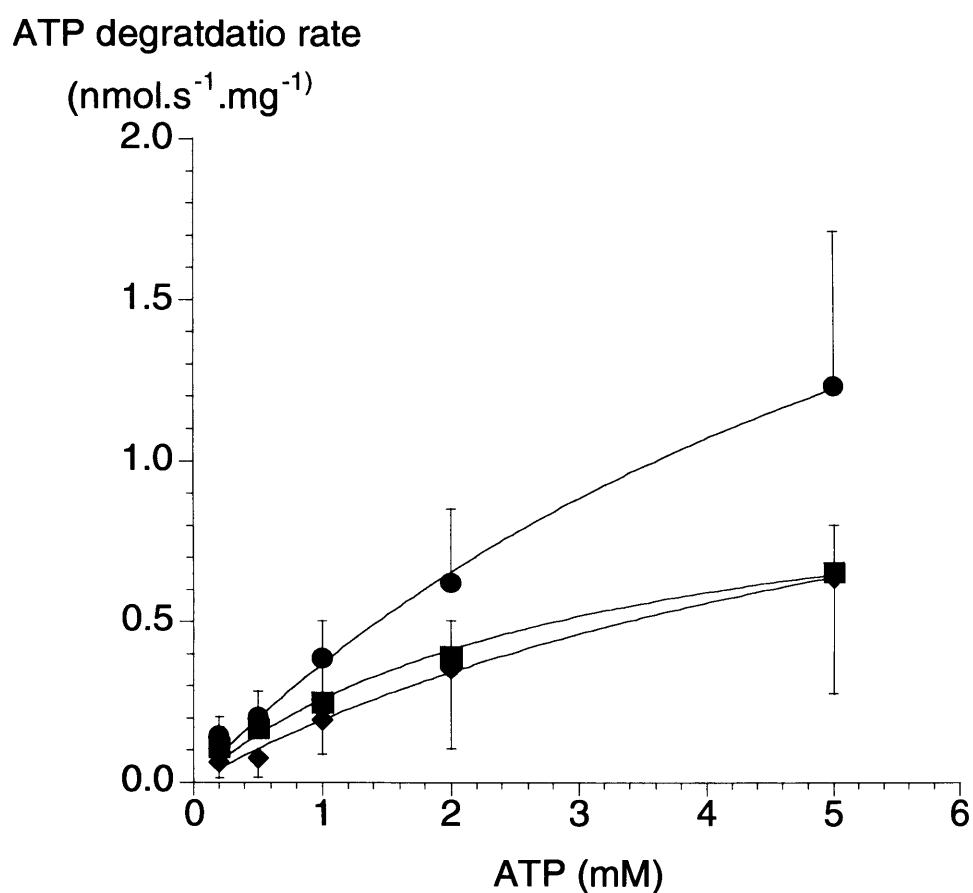


Figure 3.45 ATP degradation over a range of ATP concentrations in human detrusor from overactive bladders. Control: circles, ARL 67156 insensitive: squares, ARL 67156 sensitive: diamonds. Mean \pm standard deviation.

3.4.4 Comparison of data from stable and overactive human bladder samples

The aim of these experiments was to determine if there are any differences in the ATPase activity of stable and overactive human detrusor, in particular the ectonucleodiase activity.

When total ATPase activity was compared, Figure 3.46, there was no significant difference in the estimated V_{\max} or K_m values between the stable and overactive samples, $2.4 \pm 0.9 \text{ nmol.s}^{-1}.\text{mg}^{-1}$, $4.47 \pm 2.48 \text{ }\mu\text{M}$ and $2.0 \pm 1.0 \text{ nmol.s}^{-1}.\text{mg}^{-1}$, $3.33 \pm 1.74 \text{ mM}$, respectively. There was also no difference in the rate of ATP breakdown with 5 mM ATP.

However when comparing the V_{\max} values between groups in the presence of ARL 67156 there was a significant difference with a higher V_{\max} in stable detrusor, $1.59 \pm 0.66 \text{ nmol.s}^{-1}.\text{mg}^{-1}$ compared to overactive samples $1.1 \pm 0.4 \text{ nmol.s}^{-1}.\text{mg}^{-1}$ (Figure 3.47). The rate of 5 mM ATP breakdown was significantly greater in the stable group compared to the overactive group. There was no difference in the K_m values between the groups.

Figure 3.48 illustrates the estimated ARL 67156 sensitive fraction of ATPase activity in both stable and overactive human detrusor. There was no significant difference in the estimated V_{\max} or rate of breakdown of 5 mM ATP between the two groups.

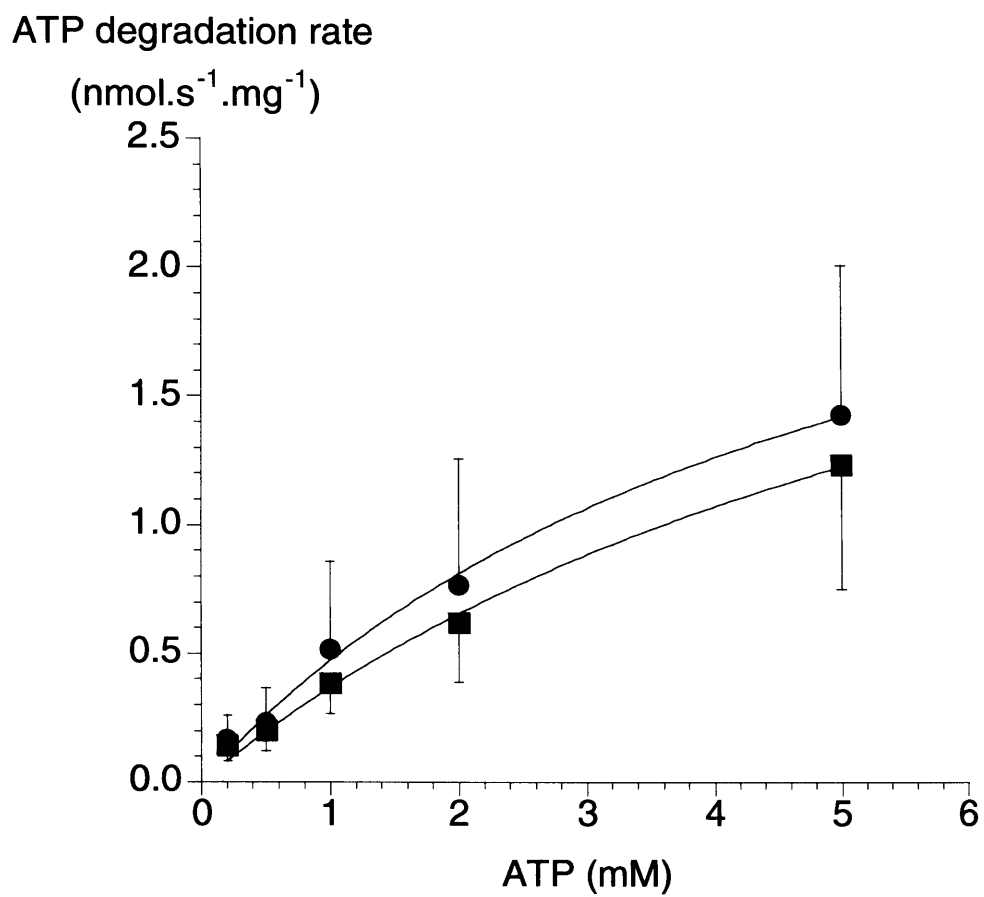


Figure 3.46 Comparison of total ATPase activity in stable (circles) and overactive (squares) human detrusor. Mean \pm standard deviation.

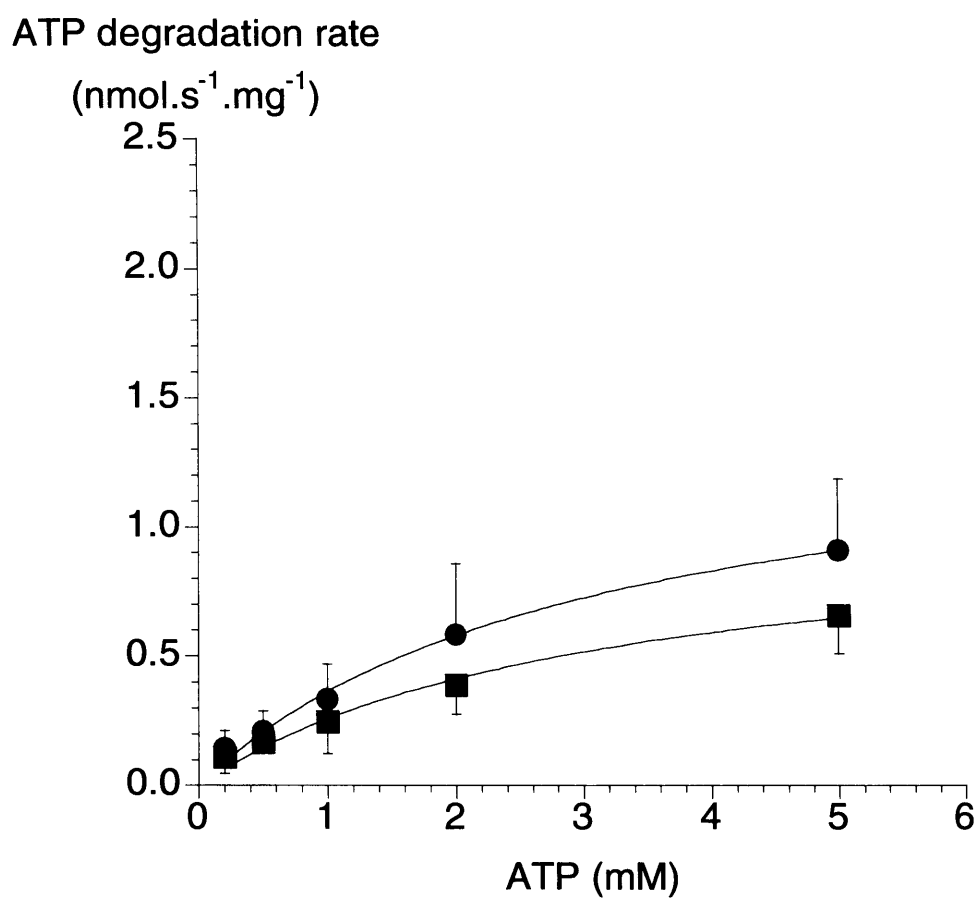


Figure 3.47 Comparison of ARL 67156 resistant ATPase activity in stable (circles) and overactive (overactive) human detrusor. Mean \pm standard deviation.

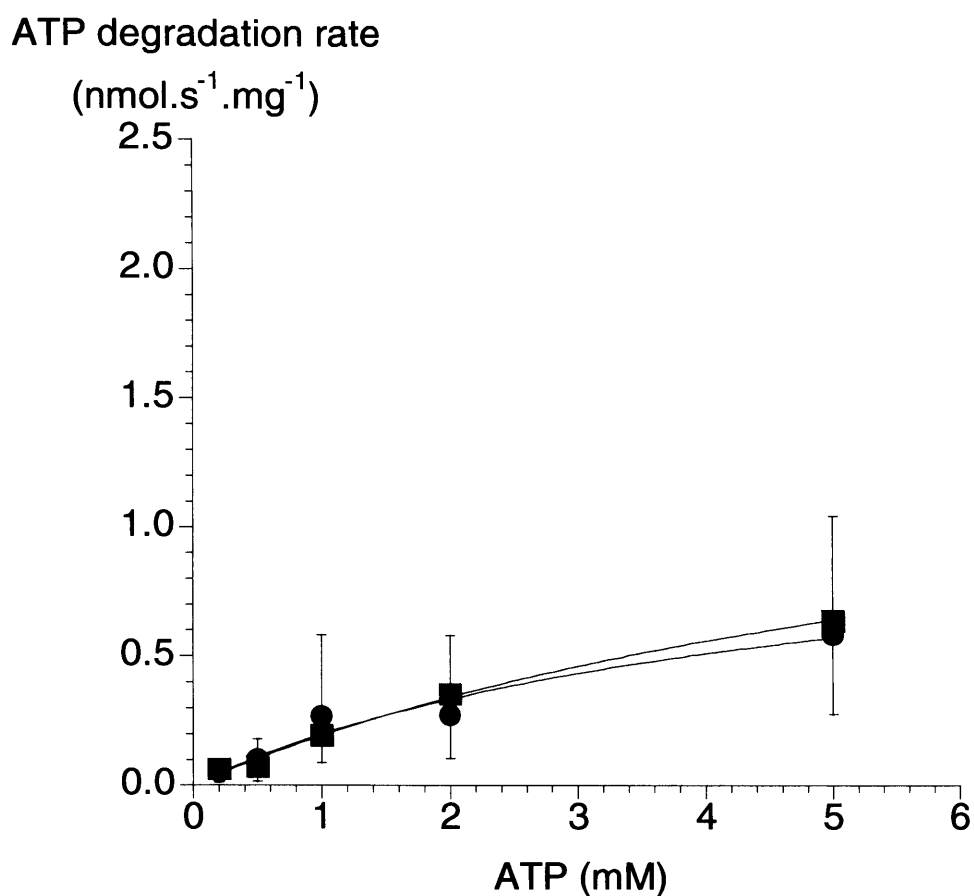


Figure 3.48 Comparison of ARL 67156 sensitive ATPase activity in stable (circle) and overactive (square) human detrusor. Mean \pm standard deviation.

3.4.5 Summary of results.

There was an ARL 67156 sensitive component of activity in all three detrusor groups indicating ectonucleotidase activity can be estimated by this method. The ARL 67156 sensitive component depends upon separate measurements in the presence and absence of ARL 67156. Therefore there will be error in the estimation of the ARL 67156 sensitive component which will be a function of the errors inherent in these two

experiments. ATPase activity has been presented in two ways, firstly through an estimation of v_{\max} from the rate vs ATP concentration curves and secondly, by presentation of data obtained at one starting concentration, 5 mM ATP. Both approaches have inherent problems the V_{\max} data are only estimations whilst the fixed concentration data represent only one sampling point. Table 3.2 shows the V_{\max} and k_m values for total ATPase, ARL 67156 resistant and ARL 67156 sensitive values for each of the three detrusor groups. Table 3.3 shows the rates for the 5 mM ATP data. Similar conclusions were drawn from both tables. Table 3.2 shows here was no significant difference between the k_m values in any of the tissue groups for the three fractions of ATPase activity. With respect to the absolute values of total ATPase activity, that measured in guinea-pig was significantly greater than in both groups of human samples; there was no difference between the two human groups. This difference was partially reflected in the ARL 67156 insensitive fraction where guinea-pig rates were significantly greater than those in samples from overactive bladders. It was also noted that the ARL 67156 insensitive rates were significantly greater in the stable bladder group compared to the overactive group. However with respect to the ARL 67156 sensitive fraction the rates were similar in all three groups irrespective of whether this was estimated from the V_{\max} data or those obtained using the 5 mM ATP data set.

This would imply that the variation in proportion of atropine resistance between the three groups is not a result of different ectonucleotidase activity and ATP breakdown. Guinea-pig detrusor has the greatest ATP component of contraction and yet a similar ectonucleotidase activity as human detrusor which shows either much less or no atropine

resistance. An hypothesis is that more ATP is released from the nerves in the guinea-pig to initiate contraction.

Table 3.4 Summary of ATP degradation by guinea pig and stable and over-active human detrusor- V_{max} and k_m data. Mean \pm standard deviation, Student's t-test *(human vs guinea-pig), ^ (stable vs overactive) $p < 0.05$

	Total		ARL 67156 insensitive		ARL 67156 sensitive	
	V_{max} nmol.s ⁻¹ . mg ⁻¹	K_m mM	V_{max} nmol.s ⁻¹ . mg ⁻¹	K_m mM	V_{max} nmol.s ⁻¹ . mg ⁻¹	K_m mM
Guinea pig	3.7 \pm 2.3	4.32 \pm 2.41	2.1 \pm 1.3	3.58 \pm 2.33	1.1 \pm 1.4	1.48 \pm 1.0
Human - stable	2.4 \pm 0.9*	4.47 \pm 2.48	1.6 \pm 0.7	3.46 \pm 1.74	1.1 \pm 0.4	4.45 \pm 2.62
Human - overactive	2.0 \pm 1.0*	3.33 \pm 1.74	1.1 \pm 0.4*^	3.06 \pm 1.43	1.2 \pm 0.9	3.33 \pm 2.48

Table 3.5 Summary of ATP degradation by guinea pig and stable and over-active human detrusor-degradation rates with initial ATP concentration 5 mM. Mean \pm standard deviation, Student's t-test *(human vs guinea-pig), ^ (stable vs overactive) $p < 0.05$

	Total	ARL 67156 insensitive	ARL 67156 67156 sensitive
Guinea pig	1.88 \pm 0.67	1.22 \pm 0.66	0.62 \pm 0.54
Human - stable	1.43 \pm 0.58	0.91 \pm 0.28	0.51 \pm 0.43
Human - overactive	1.23 \pm 0.48*	0.66 \pm 0.15 *^	0.59 \pm 0.36

3.5 REAL TIME MEASUREMENT OF ATP RELEASE

Novel ATP biosensors were used to measure for the first time the real time release of ATP from detrusor smooth muscle. The emergence of atropine resistant contractions in detrusor from overactive bladders could result from altered nerve-mediated release of ATP. The electrodes provide a useful technique to determine if there is any difference in release between the two human groups. It was not, however, possible within the time-frame of this thesis to measure the release from human samples. Guinea-pig detrusor preparations were used to establish the use of the electrodes for measuring ATP during nerve-mediated contractions. Strips were electrically field-stimulated to initiate contractions and the consequent ATP release from the preparation was measured. The aim of the experiments was to measure ATP released from nerves in the preparation during electrical stimulation.

3.5.1 Calibration

Calibration of each ATP biosensor was performed in situ with 10 μ M ATP solution. The signal obtained on the null sensor was subtracted from the signal on the ATP sensor to give the differential signal. The average voltage recorded by the ATP sensor during calibration was 4.01 ± 1.95 V. Figure 3.49 shows an example of the differential ATP calibration signal.

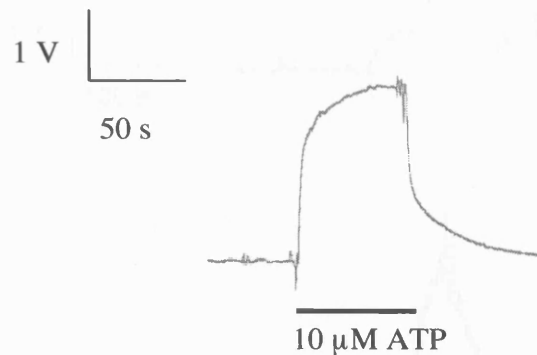


Figure 3.49 Example of ATP sensor calibration signal

3.5.2 Control contractures

To estimate the extent to which ATP released from the muscle itself, during contraction, contributes to the ATP electrode signal, the muscle strips were contracted independently of nerve mediated activation with 3 μ M carbachol and by raising the extracellular potassium concentration. The concentrations of carbachol and KCl were chosen to give comparable contraction magnitudes to those generated by nerve stimulation.

Figure 3.50 and 3.51 show examples of the tension and ATP sensor traces recorded when the detrusor strip was contracted with 3 μ M carbachol and 120 mM KCl. Both figures show that while substantial contractures were achieved the increase in voltage from the ATP sensor was only small, compared to that measured during nerve-mediated stimulation (see below).

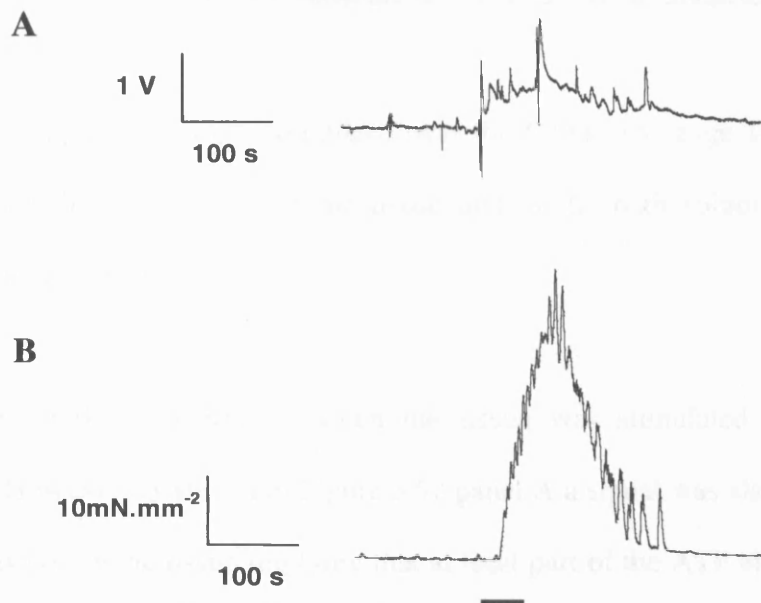


Figure 3.50 Example of A) ATP signal and B) contracture from guinea-pig detrusor strip following addition of 3μM carbachol. Solid bar indicates presence of carbachol.

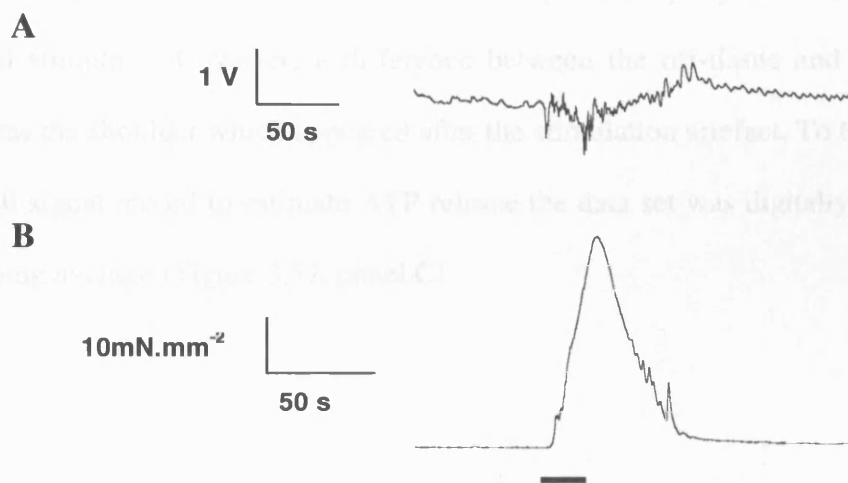


Figure 3.51 Example of A) ATP signal and B) contracture from guinea-pig detrusor strip following an increase of extracellular [KCl] to 120 mM. Solid bar indicates presence of high KCl.

3.5.3 ATP release during nerve-mediated stimulation - Initial experiments

The tissue preparations were stimulated over the frequency range 1–20 Hz, firstly with the ATP sensitive electrode off the tissue and in the bath solution, and secondly in position on the tissue.

During the initial experiments, when the tissue was stimulated a large signal was recorded. However, as shown in Figure 3.52 panel A a signal was also recorded when the sensor was not on the tissue implying that at least part of the ATP electrode signal was a stimulation artefact. Panel B shows how the initial phase of the (ATP – null) signal is oscillatory and also falls below the baseline. The stimulation artefact was greater at higher stimulation frequencies. Thus this initial phase may represent artefact from the electrical stimulus. However, a difference between the off-tissue and on-tissue ATP signal was the shoulder which appeared after the stimulation artefact. To try and improve ATP-null signal record to estimate ATP release the data set was digitally smoothed as a 5:1 running average (Figure 3.52, panel C).

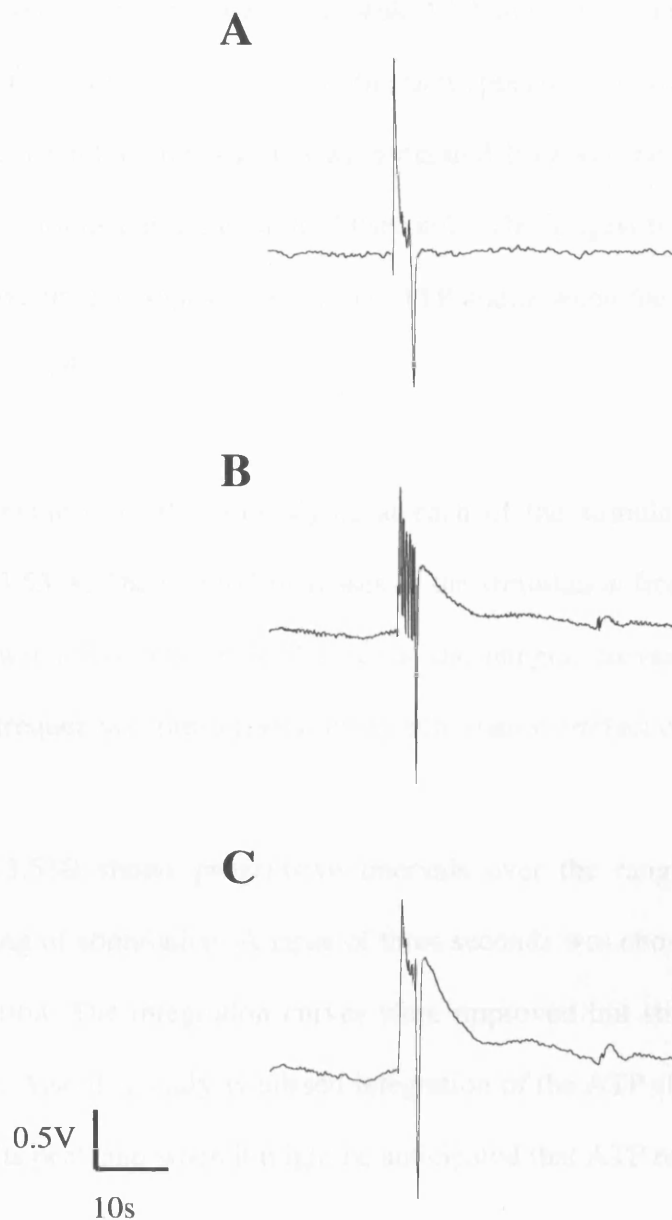


Figure 3.52 Example of ATP electrode signals during 8 Hz EFS of a guinea pig detrusor strip. A) ATP - null signal with the ATP and null electrodes off the tissue, B) ATP - null signal with electrodes on the tissue, C) ATP - null signal from part B digitally smoothed.

Once the data were smoothed they were analysed to estimate ATP release over the stimulation frequency range. The peak ATP signal that occurred during the stimulation period could not be used as a variable as it appeared this was affected by the stimulation artefact. Therefore the signal was integrated between the initiation of stimulation and then for various durations up to 30 seconds. The longest integration interval was chosen as this would encompass most of the ATP signal when the electrodes were on the tissue (Figure 3.52B).

The integration of the ATP signal at each of the stimulation frequencies is shown in Figure 3.53 A. The integral increases as the stimulation frequency is successively raised, but between zero and about 7 seconds the integral curves are unreliable, especially at higher frequencies, presumably due to stimulation artefact contamination.

Figure 3.53B shows progressive intervals over the range 3 to 30 seconds after the beginning of stimulation. A lapse of three seconds was chosen as this was the duration of stimulation. The integration curves were improved but still showed some effect of the artefact. Also this analysis missed integration of the ATP signal at the time when tension was at its peak and when it might be anticipated that ATP release would be occurring.

To include the early part of the signal, but avoid the major effects of the stimulation artefact, the signal was integrated from time zero, but the signal during the artefact was not included (Figure 3.53 C). The latter was estimated from the null electrode signal – i.e the end of the negative-going signal as in Figure 3.52A. This ATP signal in volts was

converted to ATP concentration using the 10 μ M ATP calibration, the integration of which is showed in figure 3.53D.

Although the data could be analysed and a frequency-dependence was observed in the integrated ATP signal, as shown in panel D, it was not ideal to have such a large stimulation artefact in the data or not to include a section of the data in analysis. Therefore changes to the experimental set up were made. Efforts were made to improve the experimental system to minimise the stimulation artefact.

3.5.4 Improved experimental set up

To minimise the stimulation artefact an optically-isolated constant voltage stimulator was interposed between the stimulator and the stimulating electrodes. This dramatically improved the ATP and null sensor signals as shown in Figure 3.54. Unlike previously there was no stimulation artefact when the electrode was on or off of the tissue. There was also no artefact present on the null sensor signal and when this signal was subtracted from the ATP electrode signal the consequent ATP-related signal was more evident.

As the detrusor strip was electrically field stimulated over a range of frequencies the tension and signals from the ATP sensitive and null sensor were recorded. The difference between the ATP and null sensor signals was recorded simultaneously. Figure 3.55 shows an example of raw data obtained over the frequency range 1 to 24 Hz and

demonstrates how the ATP and null signals were improved, with a lack of a stimulation artefact.

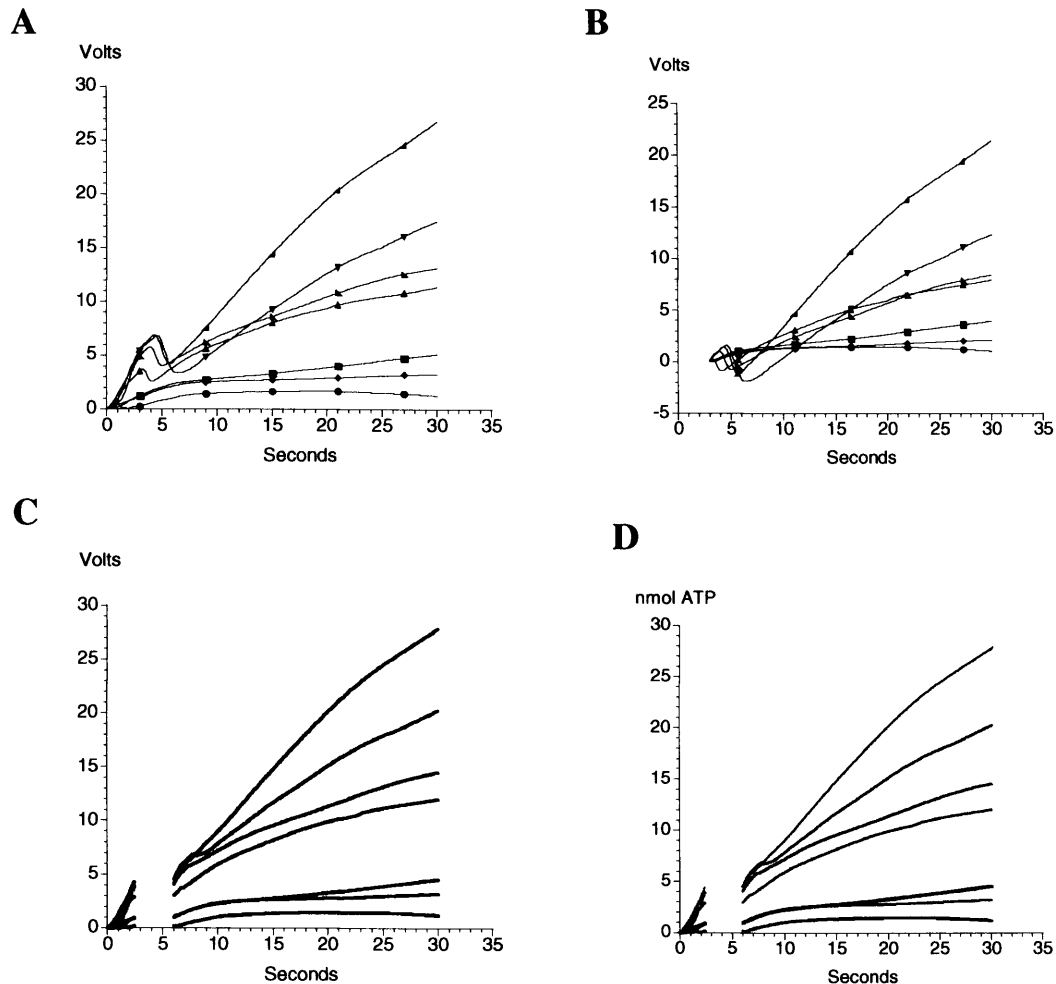


Figure 3.53 Example of integration of smoothed ATP signals following EFS of guinea pig detrusor over the frequency range 1 – 20 Hz. A) Integration from time of stimulation up to 30 seconds. B) Integration from 3 seconds after time of stimulation up to 30 seconds. C) Integration from time of stimulation up to 30 seconds but not including the signal during the stimulation artefact. D) Integration as C expressed as ATP concentration.

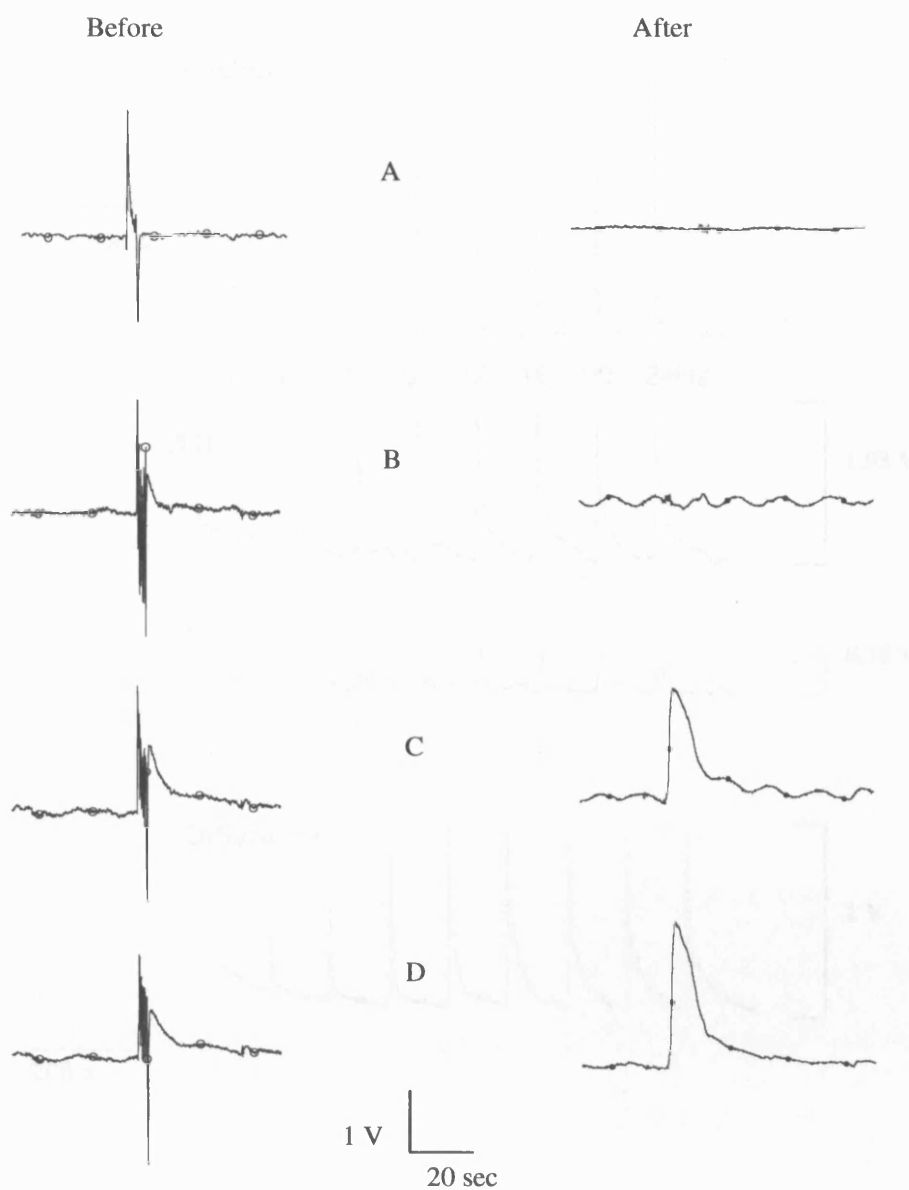


Figure 3.54 Examples of electrode signals before and after the inclusion of an optically-isolated constant voltage stimulator. A) Electrodes off-tissue signal, B) Null electrodes signal, C) ATP-electrode signal, D) ATP electrode – null electrode signal. All traces obtained at 24 Hz stimulation rate

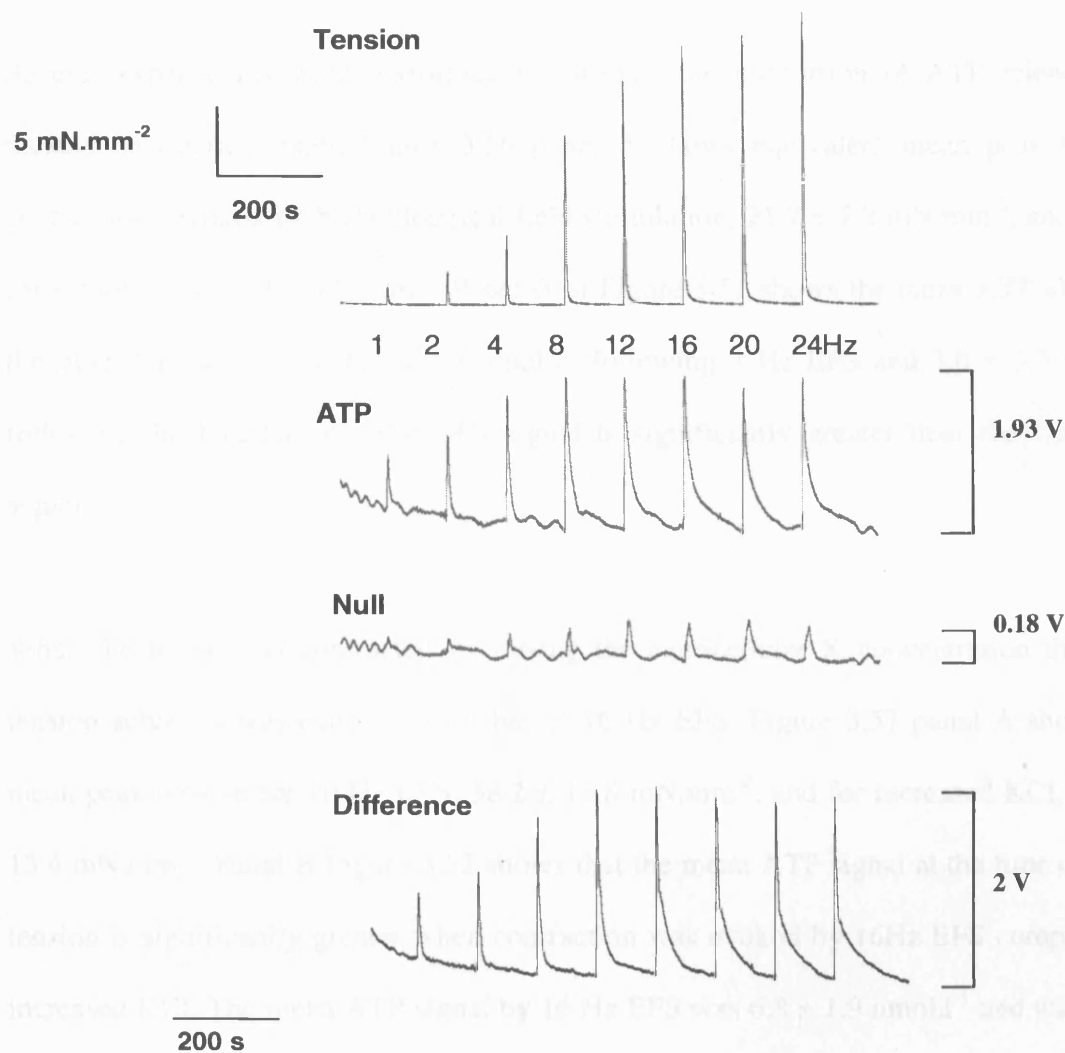


Figure 3.55 Example of raw signals obtained from null and ATP sensors, the difference of the signals and the tension recorded when a guinea-pig detrusor strip was electrically field stimulated over the frequency range 1 to 24 Hz.

3.5.5 Carbachol and KCl contractures

Several experiments were performed to estimate the proportion of ATP released by muscle contraction itself. Figure 3.56 panel A shows equivalent mean peak tension contractions evoked by 8 Hz electrical field stimulation, $21.7 \pm 7.2 \text{ mN.mm}^{-2}$, and $3 \mu\text{M}$ carbachol, $24.4 \pm 14.3 \text{ mN.mm}^{-2}$. Panel B of Figure 3.51 shows the mean ATP signal at the time of peak tension, $6.5 \pm 3.9 \text{ nmol.l}^{-1}$ following 8 Hz EFS and $3.0 \pm 3.0 \text{ nmol.l}^{-1}$ following $3 \mu\text{M}$ carbachol. The EFS signal is significantly greater than the carbachol signal.

When the tissue was contracted by raising the extracellular K concentration the peak tension achieved was equivalent to that of 16 Hz EFS. Figure 3.57 panel A shows the mean peak tension for 16 Hz EFS, $38.2 \pm 13.8 \text{ mN.mm}^{-2}$, and for increased KCl, $36.9 \pm 13.4 \text{ mN.mm}^{-2}$. Panel B Figure 3.52 shows that the mean ATP signal at the time of peak tension is significantly greater when contraction was evoked by 16Hz EFS compared to increased KCl. The mean ATP signal by 16 Hz EFS was $6.8 \pm 1.9 \text{ nmol.l}^{-1}$ and was $1.6 \pm 1.2 \text{ nmol.l}^{-1}$ by increased KCl.

EFS contractions and nerve-independent contractures had equivalent mean peak tensions the ATP signal was significantly greater for the EFS evoked contraction. These results indicate that at worst less than half of the ATP signal can be attributed to the contraction of the muscle itself.

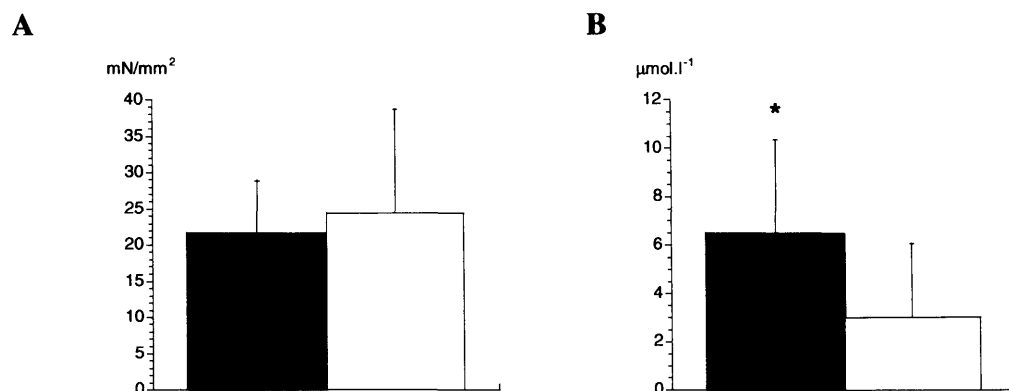


Figure 3.56 Estimation of the proportion of ATP released by the contraction of guinea pig detrusor muscle itself. A) Equivalent mean peak tension contractions evoked by 8 Hz EFS (grey) and 3 μM carbachol (white). B) ATP signal at time of peak tension of 8 Hz EFS (grey) and 3 μM carbachol (white). Mean ± standard deviation, Student's t-test $p < 0.05$.

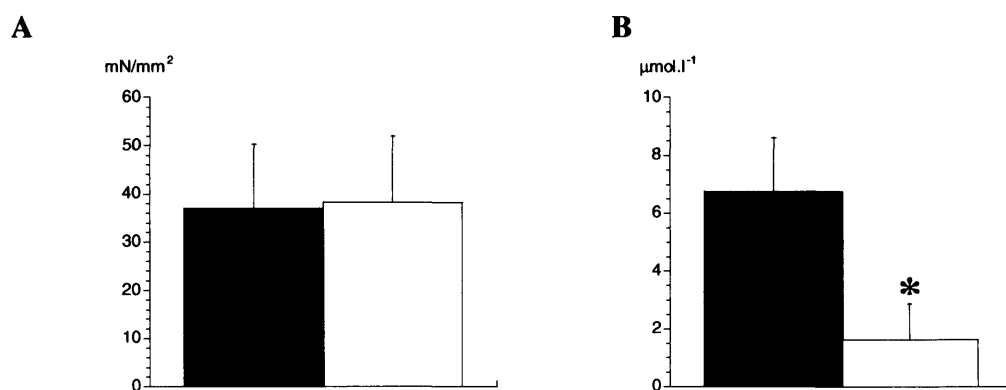


Figure 3.57 Estimation of the proportion of ATP released by the contraction of guinea pig detrusor muscle itself. A) Equivalent mean peak tension contractions evoked by 16 Hz EFS (grey) and increased extracellular potassium levels (white). B) ATP signal at time of peak tension of 8 Hz EFS (grey) and increased extracellular potassium level (white). Mean ± standard deviation, Student's t-test $p < 0.05$.

3.3.6 Frequency-dependent ATP release

The difference signal (ATP – null electrode signal) for each frequency was again integrated from time of stimulation up to 30 seconds. The average integration at each frequency is plotted in Figure 3.58. The graph shows that as the frequency increased from 1 to 12 Hz there was a large increase in the ATP integral. Further increases in stimulation frequency did not result in any further increases in the ATP integral. In fact the integral became slightly smaller at 20 Hz and then much smaller at 24 Hz.

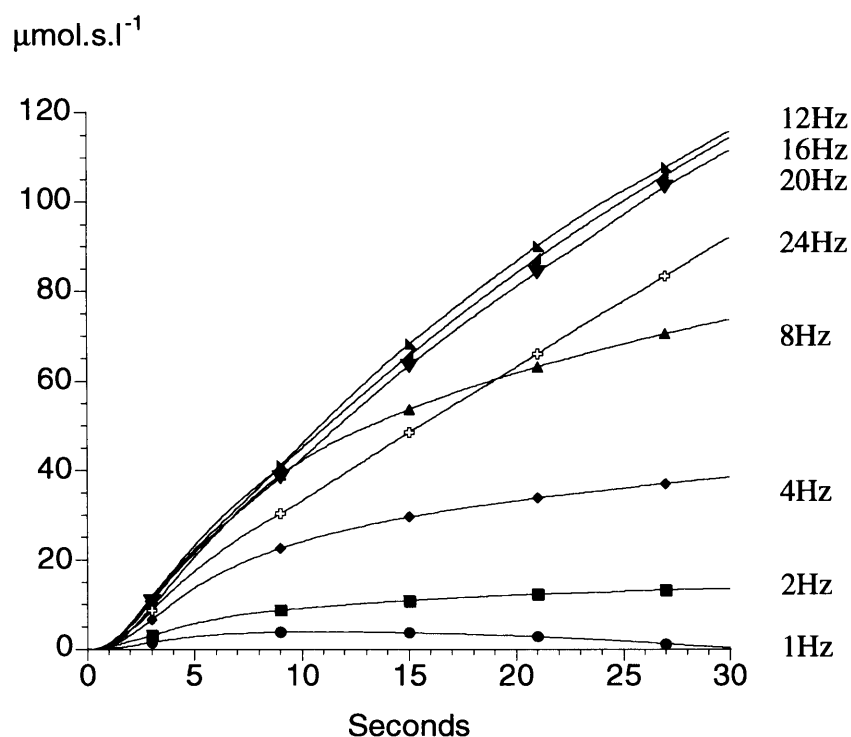


Figure 3.58 Average integration of ATP release from time of stimulation up to 30 seconds following EFS of guinea pig detrusor strips over frequency range 1 – 20 Hz. Standard deviations not plotted for clarity.

The frequency-dependence of the contractile response and the ATP electrode signal are illustrated in Figure 3.59. Average tension and the ATP integral over the first five seconds following stimulation are shown over the frequency range 1 to 24 Hz. Each curve has been normalised as a proportion of the maximal response. The ATP integral reaches a maximal response at a lower frequency compared to tension. This is confirmed by estimating the frequency required to produce half the maximal response for each variable, the $f_{1/2}$ value Figure 3.60. The ATP integral $f_{1/2}$, 4.4 ± 3.0 Hz, was significantly smaller than the tension $f_{1/2}$, 12.0 ± 2.6 Hz. This would suggest that ATP is preferentially released at lower frequencies of stimulation.

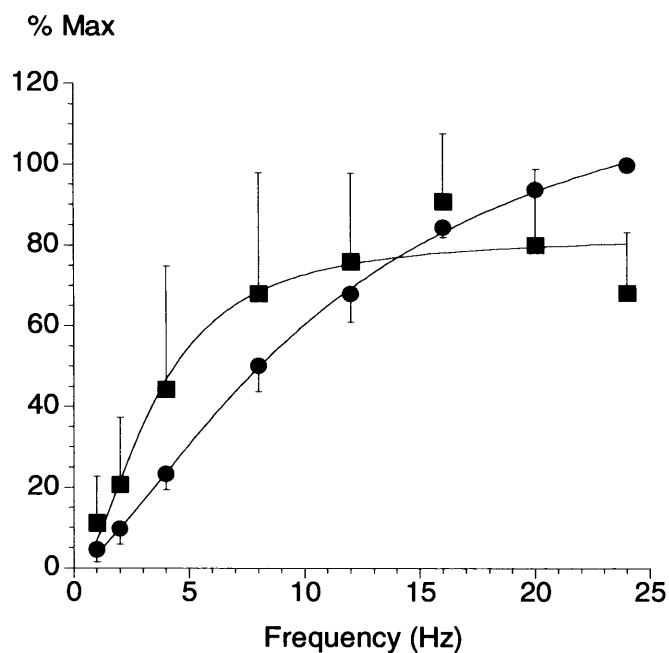


Figure 3.59 Average ATP integral and tension as a percentage of maximal response over frequency range 1 to 24 Hz. ATP signal was integrated for five seconds from time of stimulation. ATP integral: squares, tension: circles. Mean \pm standard deviation.

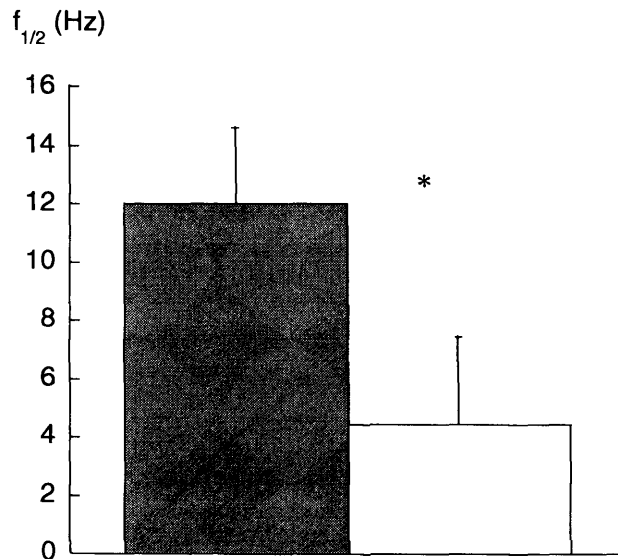


Figure 3.60 The estimated frequency required to produce half the maximal response of tension and ATP signal integrated over five seconds from time of stimulation. Tension: grey, ATP integral: white. Mean \pm standard deviation, $p < 0.05$ Student's paired t-test.

3.3.7 ATP release in the presence of atropine

In the presence of $1\mu\text{M}$ atropine the integration of the ATP signal from the time of stimulation up to 30 seconds over the frequency range 1 to 24 Hz followed a similar pattern to those in control conditions. The ATP integral increased as the stimulation frequency increased from 1 to 12 Hz and then fell as the frequency increased up to 24 Hz, Figure 3.61.

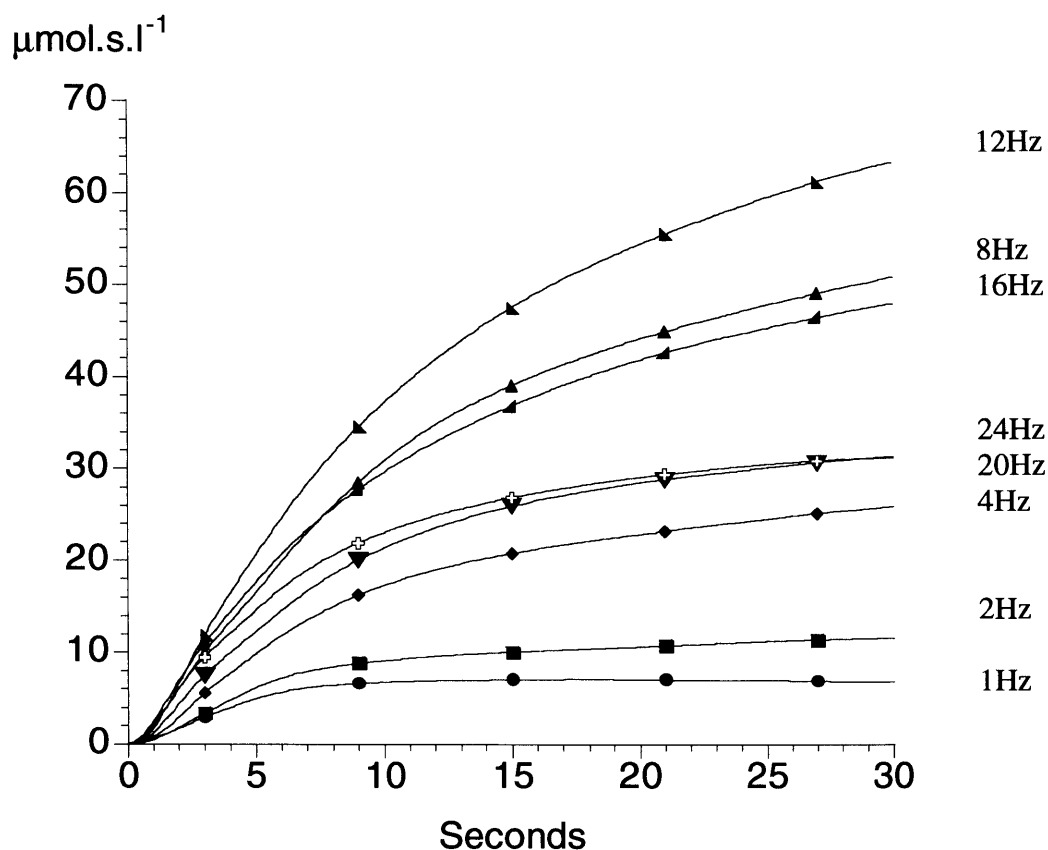


Figure 3.61 Average integration of ATP release from time of stimulation up to 30 seconds following EFS of guinea pig detrusor strips over frequency range 1 – 20 Hz in the presence of 1 μ M atropine. Standard deviations not plotted for clarity.

Figure 3.62 illustrates the frequency-dependence of the ATP release and tension in the presence of 1 μ M atropine. The average tension and five second ATP integral over the frequency range 1 to 24 Hz are shown. In the presence of 1 μ M atropine the frequency dependence of the ATP signal and tension were comparable. The maximum response reached by each variable is achieved at an equivalent frequency.

The average estimated frequencies required to produce half maximal tension and for the five second ATP integral were not significantly different 7.3 ± 3.5 Hz and 3.1 ± 1.6 Hz respectively, (Figure 3.62). This is contrast to the situation when total nerve-mediated tension was compared to the frequency-dependence of ATP release.

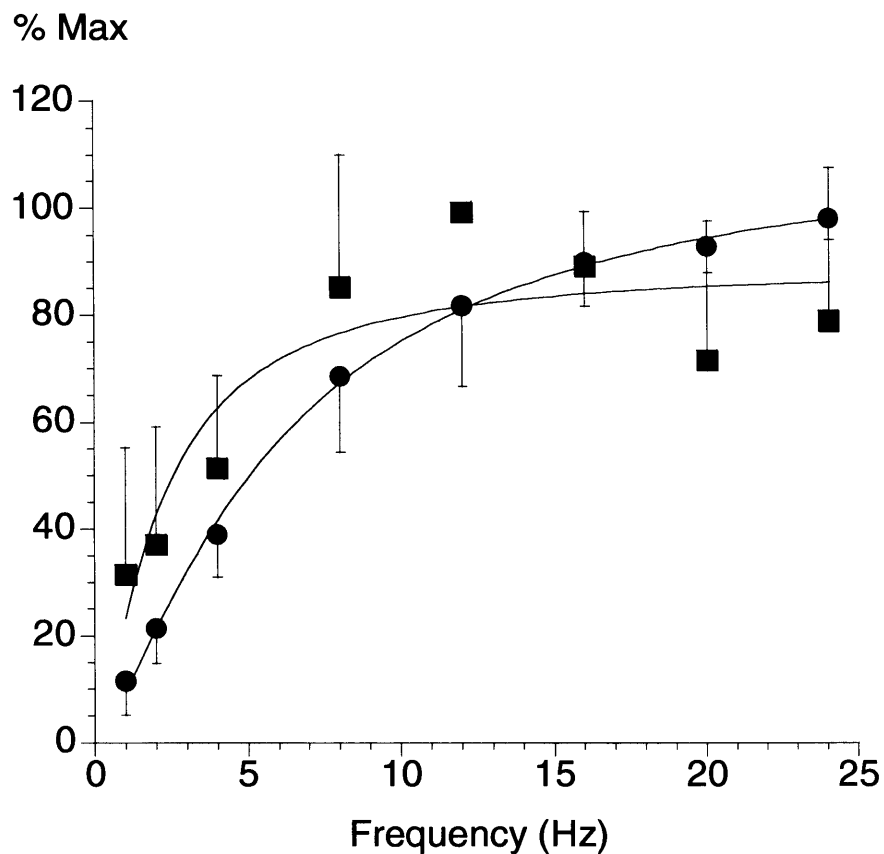


Figure 3.62 Average ATP integral and tension as a percentage of maximal response over frequency range 1 to 24 Hz in the presence of 1 μ M atropine. ATP signal was integrated for five seconds from time of stimulation. ATP integral: squares, tension: circles. Mean \pm standard deviation.

In summary, atropine caused a shift in the tension force-frequency curve to the left compared to control. The tension $f_{1/2}$ was significantly smaller in the presence of atropine. However there was no variation between the ATP signal $f_{1/2}$ in the two conditions and the values were not significantly different from the tension force-frequency $f_{1/2}$ in the presence of atropine., Figure 3.63.

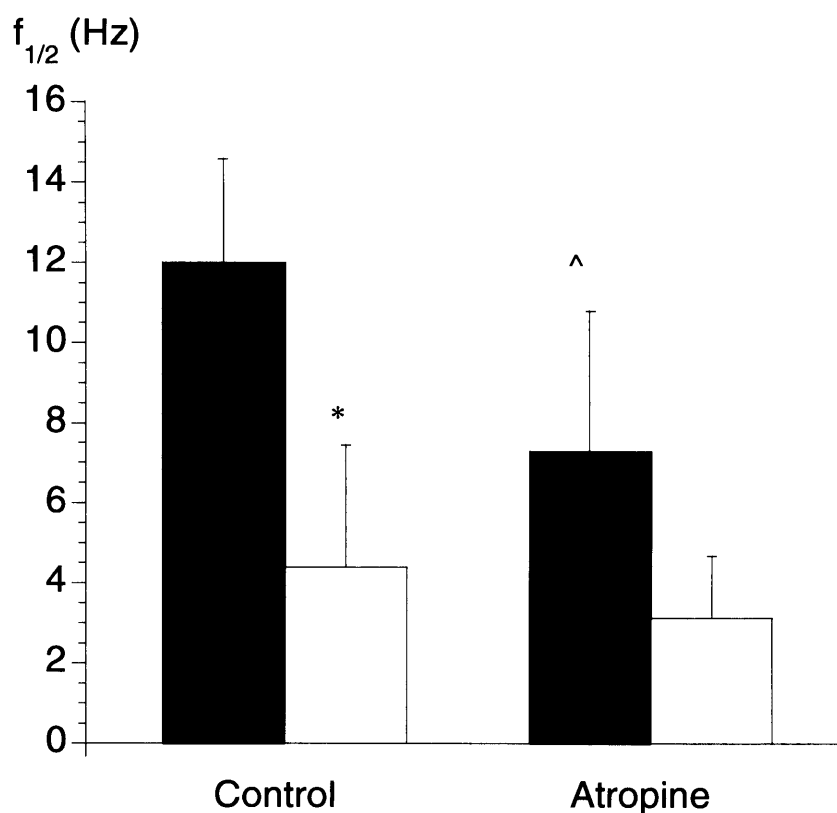


Figure 3.63 Average estimated frequency required to produce half the maximal tension and five seconds ATP integral in the presence and absence of 1 μ M atropine, tension: black, ATP: white. Mean \pm standard deviation, * $^{\wedge}$ $p < 0.05$.

Figure 3.64 demonstrates that the relationship between tension generated and ATP released is not linear, with ATP signal reaching a maximum before tension.

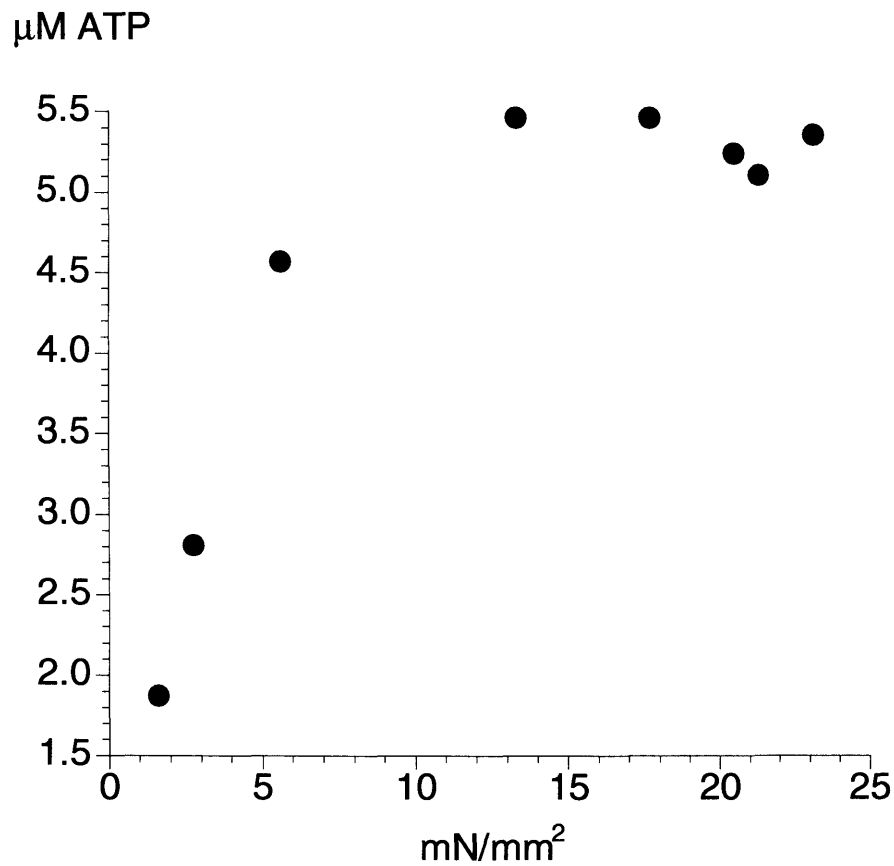


Figure 3.64 Sample plot of tension ($\text{mN} \cdot \text{mm}^{-2}$) against peak ATP (μM) obtained with electrical field stimulation over the frequency range 1 to 24Hz.

In the presence of 1 μM TTX nerve-mediated contractions and the ATP electrode signals at all frequencies were abolished.

3.5.8 Summary of results

These results show that the novel ATP sensitive microelectrode biosensors provide a useful technique to measure real-time release of ATP during nerve-mediated contraction in detrusor smooth muscle. When ATP integrals from equivalent contractions evoked by direct muscle stimulation using carbachol and 8Hz electrical field stimulation were compared the 8Hz integral was significantly greater than the carbachol. This indicates that a proportion of the ATP measured is released from stimulated nerves. The release of ATP was frequency dependent with a preferential release of ATP at low stimulation frequencies. The electrodes could now be used to determine any differences in ATP release in detrusor from stable and overactive human bladders that may contribute to atropine resistance and symptoms of overactivity.

4.0 Discussion

4.1 EXPERIMENTAL LIMITATIONS

In this thesis a number of techniques were used to investigate the role of extracellular ATP in signalling pathways concerned with neurally-mediated activation of detrusor smooth muscle. These included the measurement of isometric tension experiments with muscle strips, estimation of ectonucleotidase activity through ATP degradation, molecular studies of gene expression and measurement of ATP release using microelectrode biosensors.

4.1.1 Isometric tension experiments

Tension experiments, used to look at the effect of apyrase and ARL 67156, used small tissue preparations of approximately 1 mm in width and 4-6 mm in length. Using small strips allows for adequate superfusion of the tissue by solutions, and the fluid exchange time through the organ bath was sufficient to prevent hypoxia within the tissue. One limitation of some of these experiments was due to the expense of apyrase, such that experiments using this agent were carried out when preparations were not superfused. Instead superfusate flow was stopped and 1ml apyrase containing Tyrode's solution was added directly to the bath. When the superfusate was suspended for the addition of apyrase the duration was limited to prevent impairment of the tissue. Force-frequency

curves were comparable in flow and no-flow conditions and upon restoration of flow, indicating the tissue did not suffer any negative effects during these interventions.

There is also a possibility that the contractile response may be distorted by the amount of connective tissue in the sample muscle strip, with little or no contractile response in some samples from overactive human bladders. It was observed by microscopy that the connective tissue content varied between species and sample groups with greater amounts in human compared to guinea-pig. In some overactive human bladder strips there may also have been some denervation as the response to electrical field stimulation was poor compared to stable human and guinea-pig samples. In these instances the stimulation strength had to be increased. Any changes to the stimulation parameters may have increased the amount of direct muscle stimulation however in the majority of samples it was found that 1 μ M TTX abolished contractions so that direct muscle stimulation was generally negligible.

4.1.2 Molecular experiments

Molecular studies were used to extract RNA from human detrusor samples which were then used to determine if specific ectonucleotidase genes were expressed. Human bladder samples were limited and often small. However the sensitive techniques used require only small amounts of starting material; 30 mg of each bladder sample was used. RNA extraction was simplified by using purchased RNA extraction kits from Sigma. The kit

enables rapid extraction of RNA which is important to prevent RNA degradation that occurs once the sample is defrosted. The other consideration when preparing RNA samples is contamination. To determine the extent of degradation and contamination each sample was analysed using the Aligent 2100 bioanalyser. This enabled the samples to be visualized on a gel-like image and any samples which were degraded or contained a contaminant could be eliminated, and so reduce any inaccuracies in analysis. An additional benefit of this approach was that very small quantities, 1 μ l, of sample were required. Unfortunately a number of samples had to be eliminated at this stage from further investigation due to degradation. It is important to freeze rapidly samples of tissue in liquid nitrogen once they are removed from the bladder during the surgical procedure to prevent degradation of the RNA. However due to the location of some of the operating theatres it was not always practical or possible to freeze samples immediately.

Pre-designed and pre-optimised probe and primer sets for quantitative gene expression analysis of human genes were used to obtain the best results. Even so a number of measures were taken to ensure accuracy, including all samples being prepared in triplicate and an internal control was included in each sample. As a sample of known ectonucleotidase 1, 2, 3 and 5 expression was not included in the analysis, comparisons in expression level was made between the stable and overactive sample groups.

4.1.3 ATP degradation experiments

Experiments measuring the rate of ATP degradation by human detrusor samples were carried out to measure the activity of ectonucleotidases in the tissue. The original intention was to perform measurements on each sample before and after addition of the ectonucleotidase inhibitor ARL 67156. However due to time restraints the addition of ARL 67156 could not be performed on exactly the same piece of tissue as the control but on an adjacent piece of tissue from the same sample. This will have increased the error in calculating the ARL 67156-sensitive component of ATPase activity by the detrusor sample.

There were a number of initial problems with obtaining reproducible results from the spectrophotometer used to measure ATP-degradation products. There appeared to be cross-over between different wells of the reader-plate, particularly with samples containing higher concentrations of ATP. A number of steps were taken to reduce this error and improve the accuracy of the measurements. When the initial ATP concentration was 1, 2 or 5 mM two aliquots were taken at each time point to increase the accuracy of the measurements, and the samples were spread out as much as possible on the plates. Also an ATP standard curve was measured at the beginning of each experiment.

The k_m and V_{max} values for total ATPase and ectonucleotidase activity in the stable and overactive bladder groups were used as the derived experimental variables, and were calculated from an estimated curve fit of ATPase degradation rate as a function of initial

ATP concentration. There were a number of calculations from the raw data to the final estimations, increasing experimental errors, and it was not always possible to fit a curve of ATP degradation rate against increasing ATP concentration to obtain V_{\max} or k_m estimates for individual tissue samples. The ATPase activity due to ectonucleotidases was derived by subtracting the activity measured in the presence of ARL 67156 from the total ATPase activity of the tissue, as described above. Therefore there will be an error in the estimation of the ARL 67156-sensitive component which will be a function of the errors inherent in these two separate series of measurements. At lower initial concentrations of ATP the proportion of the ARL 67156-sensitive activity was small compared to the total ATPase activity, although they were significantly different. As the V_{\max} and k_m were estimates an alternative way of comparing the ATPase activity in the two groups of tissue samples was used. This was to compare rates of ATP breakdown at a fixed initial concentration of ATP, 5 mM. As there were no differences in the k_m between the two groups the use of a single fixed concentration to estimate ATPase rates was justified. The results and conclusions were similar with either method of analysis.

The above method was used in this study to compare ectonucleotidase activity within different experimental groups as the luciferin-luciferase assay is sensitive. However some problems with the protocol have resulted in reservations about the accuracy of the results, due to the inherent errors. If this experiment was to be repeated it is proposed that alternative methods, such as HPLC analysis, might also be used for comparison. HPLC would also enable the measurement of the particular ATP degradation products from the same samples to be measured.

4.1.4 Microelectrode biosensor experiments

The electrode used to measure ATP release from guinea-pig detrusor strips had not previously been used on electrically field-stimulated smooth muscle preparations. The experimental parameters and protocol were therefore unknown and had to be determined and optimised. However the benefits of the electrode over the other, more established methods of measuring ATP (described in the introduction) were significant, especially measuring ATP release in real-time. Calibration of the electrodes was performed *in situ* and needed to be carried out at the beginning, end and once during each experiment to account for decreasing sensitivity due to wear-and-tear of the electrode during the experiment. The effect of any interference or noise in the system was reduced by using a null electrode alongside the active electrode.

4.2 RESULTS OVERVIEW

The aim of this thesis was to study the role of extracellular ATP signalling in the neuromuscular junction of detrusor smooth muscle contractile function. The role that ectonucleotidases may have in variable ATP breakdown associated with bladder overactivity was investigated.

4.2.1 Isometric tension experiments

The contribution of attenuated ectonucleotidase activity on altering isometric tension in detrusor from some experimental groups was tested by increasing nerve-derived ATP breakdown with the addition of apyrase, a relatively non-specific ATP and ADPase. This would indicate any change in contraction that may result from changes in the activity of endogenous ectonucleotidases. The addition of apyrase resulted in a reduction of nerve-mediated force using detrusor from both human patients with overactive bladders and guinea-pig. This was in contrast to contractions elicited in human detrusor where there was no significant effect of apyrase and so no ATP component of contraction.

Upon addition of ARL 67156, an ectonucleotidase inhibitor, to guinea-pig detrusor preparations there was a reversible and significant increase in the force of nerve-mediated contraction compared to control. This again indicates that ATP is released during nerve-mediated stimulation. An additional experiment would have been to test the effects of ARL 67156 on nerve-mediated contractions in both the human detrusor sample groups however this was not possible due to the expense and lack of availability of the inhibitor.

4.2.2 Ectonucleotidase expression

Although it is believed that the breakdown of ATP in the neuromuscular junction in detrusor is by ectonucleotidases there was no previous demonstration of their gene expression in this tissue. Therefore molecular studies were performed to identify ENTPDase 1, 2, 3 and 5 expression and to determine if there were any differences in expression levels. RNA was extracted from samples of detrusor from patients with stable and overactive bladders. Following reverse transcription to cDNA, Taqman RT-PCR was performed on the samples to determine a threshold cycle number for each of the genes of interest. The results showed that mRNA to all four enzymes was present in both sample groups. There were however some differences between the groups with higher levels of ENTPDase 1 and 3 in stable compared to overactive bladders. There was also a difference in the relative levels of expression, with a significantly higher level of ENTPDase 1 compared to the other three.

4.2.3 Ectonucleotidase activity

ATP degradation was measured to estimate directly any variation of ATPase activity in the different experimental groups. A preliminary study, with small numbers of preparations, indicated that there was a reduction of ectonucleotidase activity in detrusor samples from overactive bladders compared to those from stable bladders (Harvey et al, 2002). Degradation of five concentrations of ATP by detrusor samples over a period of

30 minutes was measured in the presence and absence of ARL 67156. ATP concentrations of aliquots taken at specific time points were measured using a luciferin-luciferase assay. The rate of degradation of ATP increased with greater initial concentrations of ATP in both sample groups and the presence of ARL 67156 reduced all rates of degradation. There were no significant differences in total ATPase activity between detrusor samples from the stable and overactive bladder groups in the rate of ATP breakdown with an initial concentration of 5 mM, or the k_m or V_{max} values. . The ARL 67156-resistant fraction of activity was significantly greater in detrusor from stable bladders compared to overactive bladders. However there was no difference in the ARL 67156-sensitive activity. Thus no evidence was found for a difference in unit ectonucleotidase activity in detrusor samples from patients with stable or overactive bladders.

4.2.4 Real time ATP release

To try to measure the real-time release of ATP from stimulated nerves in detrusor smooth muscle novel ATP microelectrode biosensors were used. An experimental set-up and protocol was designed to determine the utility of the novel electrodes in this tissue. It was possible to record extracellular ATP signals following direct muscle stimulation with carbachol and electrical field stimulation. All nerve-mediated signals were abolished in the presence of TTX and reduced by atropine although to a lesser extent than the force of contraction. The relationship between ATP release and stimulation frequency was

investigated. The ATP signal reached a maximal response at a lower frequency compared to tension. Comparing the ATP integral obtained from equivalent mean peak tension contractions evoked by electrical field stimulation and 3 μ M carbachol it was shown that a proportion of the ATP signal can be attributed to ATP released from nerves following stimulation.

4.3 ATP AND NERVE-MEDIATED CONTRACTIONS IN DETRUSOR SMOOTH MUSCLE

Nerve-mediated contractions and force-frequency relationships of human and guinea-pig detrusor smooth muscle were assessed and the effect of altering ATP degradation on contractions was investigated.

4.3.1 Contractile properties of human and guinea-pig detrusor to electrical field stimulation

The magnitude of force generated when normalised per unit cross-section of preparation from nervemediated contractions of detrusor preparations from human, both stable and overactive, and guinea-pig bladders was similar at their half-maximum stimulation frequencies, 20 and 8 Hz respectively. This implies the contractility of the detrusor

muscle using this method of stimulation is similar between the two species and between control and pathological sources of human tissue. There was however a difference in the force-frequency relationship between the two species as there was a difference in the half maximal stimulation-frequency ($f_{1/2}$). Human detrusor required a higher stimulation frequency, with the $f_{1/2}$ for stable bladder samples being 15.9 ± 7.0 Hz, and overactive bladder samples were not significantly different from stable bladders with an $f_{1/2}$ of 19.9 ± 11.2 Hz. However guinea-pig detrusor had a value of 9.5 ± 3.5 Hz. These results were comparable to those of previous studies (Ikeda, 2006; Harvey et al, 2002; Bayliss et al, 1999). This variation may be due to a difference in the neurotransmitters released by the two species. Guinea-pig detrusor has two significant components to contraction, ATP and acetylcholine, compared to just acetylcholine in human (see Introduction – section 1.4). This was also evident by the significant atropine-resistance observed in guinea-pig preparations. This difference in $f_{1/2}$ value between the two species is compatible with the hypothesis that ATP is released at lower stimulations frequencies than is Ach (See Discussion – section 4.8). There was no significant difference in the maximum force or $f_{1/2}$ frequencies of nerve-mediated contractions in the detrusor samples from stable and overactive bladders. However it may be argued that if there is some atropine-resistance in detrusor from overactive human bladders that this group should have an intermediate $f_{1/2}$ between guinea-pig and stable human samples. In this study overactive human bladder samples are contained within one sample group however not all samples showed atropine resistance, 11 of the 28 tested, which may explain the similar $f_{1/2}$ of this group compared to stable samples.

It has been shown in a number of other species that two phases to nerve mediated contraction can be seen, for example in rabbit bladder (Zhao et al, 1993) and rat bladder (Yu et al 1996). The initial phase was due to ATP activating rapid opening of P2X₁ ligand-gated receptors which resulted in an influx of Ca²⁺. The second, later phase was dependent on acetylcholine stimulating M₃ receptors activating slower, second messenger pathways to release intracellular Ca²⁺. This bi-phasic response was not often observed in human and guinea-pig preparations, perhaps because the wave-form was dampened by the visco-elastic properties of the extracellular matrix which in human samples in particular is a significant proportion of the tissue mass.

4.3.2 Effect of altered ATP breakdown on nerve-mediated contractions

In detrusor muscle strips from guinea-pig and overactive human bladders 10 U.ml⁻¹ apyrase caused a significant and reversible reduction in the magnitude of nerve-mediated contractions elicited at 16 Hz and 24 Hz respectively. Apyrase (10 U.ml⁻¹) was also shown to significantly reduce EFS contractions in rat tail arteries to a similar extent to that seen in guinea-pig detrusor (Fukumitsu et al, 1999). In contrast to guinea-pig and overactive human bladder samples apyrase had no effect on preparations from stable human bladders.

Apyrase is a non specific ATP and ADPase that would, along with endogenous ectonucleotidases degrade ATP in the neuromuscular junction. This would support there

being an ATP component of contraction in both guinea-pig and overactive human bladder as addition of apyrase would increase breakdown and limit the amount of ATP able to activate P2X receptors on the muscle membrane to initiate a purinergic component of contraction. This is corroborated by the lack of apyrase effect in stable bladder samples and implies there is no ATP component to contraction. This lack of action of apyrase in the stable human bladder samples is not due to an inability of the muscle to respond to purinergic agonists as ATP and ABMA added to the superfusate have been shown to elicit contractions. (Bayliss et al, 1999). ATP released by motor nerves must therefore be hydrolysed completely by ectonucleotidases and explain the lack of effect of apyrase in this sample group.

There was also a positive correlation between apyrase-induced reduction of contraction and the proportion of atropine resistance. As atropine resistance increased so did the extent of reduction of contraction by apyrase. If ectonucleotidase activity was reduced in the overactive bladder, increasing ATP breakdown by addition of extra ATPase activity should reduce the atropine-resistant component of contraction observed in overactive but not stable human bladders.

The effect of enzyme activity was also investigated through the addition of the ectonucleotidase inhibitor ARL 67156. ARL 67156 inhibits ectonucleotidase activity with a pIC_{50} of 4.62 in human blood cells (Crack et al, 1995) and 5.1 in the rat vas deferens (Khakh et al, 1995). In this study 100 μ M ARL 67156 significantly potentiated nerve-mediated contractions in guinea-pig detrusor strips. Higher concentrations of ARL

67156 were not used as it is also a weak antagonist at P2X receptors (Crack et al, 1995) which would tend to reduce the force of contraction and thus confound the experimental data. By inhibiting the breakdown of ATP released from the motor nerves, more ATP was able to activate contraction. This hypothesis was further supported by the significant increase in contraction by ARL 67156 in the presence of atropine. The effect of ARL 67156 in this thesis is comparable to another study investigating the effect of the inhibitor in guinea-pig bladder (Westfall et al, 1997). In this study ARL 67156 was also shown to enhance contractions by exogenous ATP but not those to the non-hydrolysable ATP analogue, ABMA. This is consistent with ARL 67156 inhibiting breakdown of ATP, and not affecting directly smooth muscle contractile function.

It would be of interest to determine the effect of ARL 67156 in stable and overactive human detrusor. Inhibition of ectonucleotidase activity in stable detrusor samples should produce an increase of tension of nerve-mediated contractions as a result of a reduction of ATPase activity. This would provide support for the hypothesis of a reduced ectonucleotidase activity to generate atropine-resistance in some overactive bladders. If tension were increased when ARL 67156 was applied to bladder preparations from stable and overactive, this would provide further evidence that ATP is released on nerve-stimulation in human detrusor.

4.3.3 Atropine-resistant nerve-mediated contractions

Atropine-resistant contractions are evoked by non-cholinergic neurotransmitters and it has been established that in the bladder this neurotransmitter is ATP (Burnstock et al, 1978). This atropine-resistance is seen in detrusor from most animal species with the exception of humans and old world monkeys, where acetylcholine is the sole neurotransmitter (Kinder and Mundy, 1985). Atropine-resistance was observed in all guinea-pig detrusor samples, atropine reduced contractions by 41.9 % which is similar to other studies (Creed et al, 1991). Also consistent with previous work was the lack of atropine-resistance observed in all but three of the 25 stable bladders samples (Sjogren et al, 1982).

There were atropine-resistant contractions in a number of the detrusor samples from overactive human bladders. Greatest atropine-resistance in overactive bladders has previously been observed in detrusor samples from those with concomitant obstruction (Ikeda, 2006; Bayliss et al 1999). The overactive group in this thesis did not contain any obstructed bladder samples and so the extent of atropine-resistance would have been smaller than observed in other studies. Atropine-resistance has been observed in other bladder pathologies including interstitial cystitis (Palea et al, 1993), although the mechanism for the phenomenon has not been investigated in this group.

Reasons for the emergence of atropine-resistance in some disease states, as discussed in the Introduction, could include alterations in neurotransmitter release, sensitivity of the

smooth muscle receptors and reduced ectonucleotidase activity. A reduced breakdown of ATP has been proposed (Harvey et al, 2002) where there was decreased ectonucleotidase activity in overactive bladder. There has been evidence of change in receptor expression in some pathologies including interstitial cystitis (Birder et al 2004), urge incontinence (Moore et al, 2001) and an increase in P2X receptors was observed in men with outlet obstruction compared to control patients (O'Reilly et al., 2001). It is unlikely that atropine-resistance is due to a change in the sensitivity of the smooth muscle to ATP or Ach, as demonstrated by Wu et al (1999). It has also been suggested that atropine resistance increases naturally with ageing (Yoshida et al, 2001), but the reason for this is unknown.

4.4 E-NTPDase 1, 2, 3 AND 5 EXPRESSION

Nerve-stimulated ATP release from motor nerves in detrusor smooth muscle is degraded by ectonucleotidases bound to the surfaces of cells within the neuromuscular junction. The specific ectonucleotidase enzymes involved in human detrusor have not been identified. Gene expression assays were used to investigate the presence of E-NTPDase 1, 2, 3 and 5 in 18 human detrusor samples, comprising samples from both stable and overactive bladders.

The data showed that all four of the E-NTPDase were present in all human detrusor samples. The human samples were divided into stable and overactive bladder groups to

compare the relative quantity of each enzyme. There were no differences between the samples groups in the levels of E-NTPDase 2 and 5. However there was a significant reduction in overactive bladder samples compared to stable ones in the level of both E-NTPDase 1 and 3. When comparing the levels of the E-NTPDases to each other the relative quantity of E-NTPDase 1 was considerably greater than the other three. Thus any reduction in E-NTPDase 1 will be the significant one in terms of overall E-NTPDase expression.

E-NTPDase 2 has the greatest preference for ATP, over other nucleotides, whilst E-NTPDase 3 has only a slight preference for the nucleotide (Zimmermann, 2001). However the levels of these two enzymes were significantly lower than those of E-NTPDase 1 which hydrolyses ATP and ADP equally well. It is possible that the levels of E-NTPDase 1 are higher because it rapidly degrades ATP to AMP compared to E-NTPDase 2 and 3 which produce the intermediate product of ADP which is then slowly degraded to AMP (Vorhoff et al, 2005). ADP is an agonist for some purinergic, P₂, receptors present in the neuromuscular junction, therefore greater levels of E-NTPDase 1 would prevent activation of P₂ receptors by ADP and any effect this may have on detrusor contraction. As E-NTPDase 5 is bound to intracellular membranes and has a preference for nucleoside diphosphates it is unlikely that it would have any effect on breakdown of nerve-mediated released ATP.

A reduction in the relative quantity of the E-NTPDase 1 and 3 gene expression in the overactive bladder compared to stable bladder may have an implication in the breakdown

of the ATP released from motor nerves during contraction particularly as there was a reduction in the most abundant enzyme, E-NTPDase 1. With lower levels of enzymes there may not be sufficient hydrolysis of ATP to prevent it initiating contraction. It is in overactive bladders where the reduced E-NTPDase levels were observed that a purinergic component of contraction is implicated.

E-NTPDase 8 is also located on the cell surface with an extracellular facing catalytic site (Robson et al 2006) and hydrolyses ATP and ADP. The cloned human E-NTPDase 8 exhibits a preference for ATP, hydrolysing ADP poorly (Knowles and Li, 2006). Therefore this enzyme may be involved in the breakdown of ATP released from nerves in the detrusor. The levels of E-NTPDase 8 were not investigated in this thesis as a pre-optimised expression kit was not available for this enzyme. Therefore it is not known if it is present in detrusor or if there is any difference in its expression between the two bladder groups.

It should be taken into account that although the data shows a reduction in E-NTPDase 1 and 3 this is only at the gene level. The amount of enzyme at the protein level and the activity levels of the enzymes must be considered when drawing firm conclusion about the impact of these changes on ATP breakdown in physiological conditions.

4.5 ECTONUCLEOTIDASE ACTIVITY

In contrast to stable human bladder detrusor, samples from patients with overactive bladders have a purinergic component to nerve-mediated contraction shown by atropine-resistance. Experiments in this thesis tested the hypothesis that this was due to a reduced ectonucleotidase activity in overactive bladder. This would provide evidence to indicate changes in ATP breakdown were responsible for atropine-resistance in some bladder pathologies.

The data was presented in two ways: V_{\max} and k_m estimates of ATPase activity; and breakdown rates obtained with an initial starting concentration of 5 mM ATP. As the two representations gave equivalent results only V_{\max} and k_m data is discussed.

Total ATPase activity was significantly greater in detrusor from guinea-pig compared to that from humans, whilst the activity in stable and overactive bladders was comparable. The addition of ARL 67156 did not alter the k_m values for each group, but did significantly reduce the estimated V_{\max} for all groups. This indicates the presence of ectonucleotidases in both guinea-pig and human detrusor smooth muscle. The estimated ARL 67156 resistant V_{\max} for overactive bladders was significantly lower than that for the stable bladders and guinea-pig groups which were similar. However the ARL 67156 sensitive fraction of the ATP breakdown rate which was taken to be the ectonucleotidase rate only was not significantly different between any of the three detrusor groups.

This is in contrast to the previous study where the ARL 67156-sensitive fraction was smaller in samples from overactive bladders compared to those from stable bladders (Harvey et al, 2002). Comparing the results of the two studies the V_{\max} estimates for total, ARL 67156 resistant and ARL 67156 sensitive ATPase activities were very similar in the stable groups.

There was a varying proportion of atropine-resistance in detrusor from guinea-pig, and stable and overactive human bladders but an equivalent ectonucleotidase activity. This would indicate that reduced ATP breakdown is not responsible for atropine-resistance. The greatest degree of atropine-resistance was observed in guinea-pig detrusor, with much less resistance in overactive bladders and none in stable bladders. It may be hypothesised that the ATP component of contraction in guinea-pig is due to an increased release of ATP following nerve stimulation. If this is so the emergence of an ATP component of contraction in detrusor from overactive bladders may be the result of altered nerve-mediated ATP release, with an increase in release compared to stable bladders.

The proportion of atropine-resistance in human samples used for this set of experiments was not determined due to their relatively small size which would have precluded both sets of measurements being carried out on the same biopsy sample, and this would be of advantage in any future experiments of this type.

The experiments in this thesis measured the activity of membrane-bound ectonucleotidases within the detrusor. ATP breakdown following release from stimulated motor nerves may not be due to these enzymes alone. There is evidence to show that in the vas deferens of guinea pig a soluble ectonucleotidase is released upon nerve stimulation (Todorov et al, 1997). It may therefore be necessary to also stimulate the tissue when measuring degradation of ATP to include the contribution from this possible source also.

4.6 ALTERED ECTONUCLEOTIDASE ACTIVITY

Although results from this study show that there was no significant difference between ectonucleotidase activity in stable and overactive bladder, gene analysis showed there was a reduction in the levels of some E-NTPDases in overactive bladder which may effect ATP breakdown. It is important to consider what could alter the activity of ectonucleotidases in physiological conditions. The activity could be affected by conditions in the extracellular space, by expression of a less active form of the enzyme or a reduced production.

Maximal activity of E-NTPDases 1, 2, 3 and 8 are dependent on Ca^{2+} and Mg^{2+} ions in the millimolar range and they are inactive in their absence (Robson et al, 2006). However it is unlikely that these ions would differ between stable and overactive bladders. Activity is also greater at alkaline pH values (Zimmermann, 2001) and may therefore be reduced

by acidosis. Blood flow to the detrusor can be reduced during filling and spontaneous contractions particularly when there is detrusor hypertrophy such as in obstructed bladders (Azadzoi et al, 1996; Batista et al, 1996) In these conditions reduced blood flow can cause a decrease in tissue partial oxygen pressure and acidosis (Lin et al, 1995; Nielsen, 1995). Furthermore, acidosis by raising extracellular PCO₂ content increases the force of contraction (Liston et al, 1991) which may in part be due to a reduced ectonucleotidase activity. However it is not known if these effects would persist in isolated tissue.

Potential splice variants for E-NTPDase 1 and 2 have been isolated (Zimmermann, 2001). Any alteration of structure may modify the substrate specificity and activity of the enzymes particularly if the pyrase-conserved regions are effected. Glycosylation and surface location of the enzymes has also been shown to be crucial to activity (Zhong et al, 2001).

The cause of altered expression of ectonucleotidases in detrusor is unknown but has been noted in other situations. The enhanced expression of E-NTPDase 1 in endothelial and mesangial cells is downregulated by glucocorticosteroids (Kapojs et al, 2004) and altered expression has also been observed human melanomas (Dzhandzhugazyan et al, 1998).

Altered ectonucleotidase activity will not only effect the concentration of ATP within the neuromuscular junction and its ability to initiate contraction, but also the generation of its

breakdown products such as ADP, AMP and adenosine. This is important because any of these products can act on pre or post-junctional receptors to modulate contraction. P2Y receptors can be activated by ADP to initiate relaxation, reduced ATPase activity may lead to a transient increase in ADP and reduced contractile activity. Adenosine has been shown to auto-regulate neurotransmitter release through activation of pre-junctional P1 receptors. Adenosine and an A1 selective receptor agonist, CPA reduced the overflow of stimulated ATP release in the guinea-pig vas deferens (Driessen *et al*, 1994). Adenosine has also been shown reduce the strength of nerve-mediated contractions in both guinea-pig and human detrusor (Ikeda *et al*, 2003).

4.7 ECTONUCLEOTIDASE INHIBITION

There has always been a problem with inhibiting ectonucleotidase activity due to a lack of potent and selective inhibitors that do not have an effect on purinergic receptors. ARL 67156 is thought to be the only selective inhibitor with minimal effect at P2X receptors. However a recent study has shown that ARL 67156 inhibits E-NTPDase 1 and 3 but not E-NTPDase 2 (Iqbal *et al*, 2005). This means that if E-NTPDase 1, 2 and 3 are present in detrusor it cannot be concluded that all ectonucleotidase activity is inhibited. The implications for this study are that there will possibly remain some ATP breakdown in the presence of ARL 67156 in tension experiments. Also the ARL 67156 sensitive fraction in ATP degradation studies will not give a rate for complete ectonucleotidase activity. This is particularly true as it is E-NTPDase 2 that has the highest preference for

hydrolysing ATP. However as gene expression analysis showed in this thesis the most abundant E-NTPDase present in the detrusor samples was E-NTPDase 1 and therefore any continued activity of total E-NTPDase activity in the experiments is likely to be small.

Any future work may consider the use of new inhibitors that have recently been described for E-NTPDase 1, 2 and 3 termed polyoxometalates (Muller et al, 2006). They are significantly more potent than ARL 67156. The polyoxometalates are anionic complexes containing transition metal ions that are stable in aqueous solutions at biological pH values. The inhibitors vary in their selectivity for E-NTPDase 1, 2, or 3 and thus it may be possible to inhibit E-NTPDase 2 and 3 but not the highly expressed E-NTPDase 1 (Muller et al 2006). The affinity for these inhibitors for purinergic receptors is limited to P2Y₁₂ studies and although only moderate binding was observed the usefulness of these inhibitors in contractile experiments is unknown.

4.8 NERVE-MEDIATED ATP RELEASE

Atropine-resistance in overactive bladder has been observed in this thesis and by a number of other authors (Sjogren et al, 1982; Bayliss et al, 1999). One possibility for the emergence of this resistance is an alteration in the release of ATP following stimulation of nerves within the detrusor. As there is a purinergic component of detrusor contraction

in guinea-pig it was used as a suitable model to optimise the use of a novel ATP microelectrode biosensor that is a sensitive technique to measure real-time release of ATP. Future studies may then be able to use the electrodes to investigate the theory of altered ATP release in overactive bladder.

When guinea-pig detrusor muscle strips were electrically field-stimulated at a frequency range of between 1 and 20 Hz it was possible to record an ATP signal from the electrode. When the signals were integrated over 5 seconds the amount of ATP released increased with increasing frequency of stimulation. Other studies that have investigated the release of ATP from rabbit (Ferguson et al, 1997) and human and porcine (Kumar et al, 2004) bladder using the luciferin-luciferase assay have shown that the predominant release of ATP is from the urothelium. In this thesis the urothelium was removed from all guinea pig strips studied. Another potential source of ATP release other than nerves is from the activated smooth muscle cells. Stimulation-dependent release of ATP in the mouse phrenic nerve-hemidiaphragm preparation was partly postsynaptic (Vizi et al, 2000) and in the same preparation in the rat it was estimated that over 60% of released ATP is derived from muscle fibres (Santos et al, 2003). ATP is released from the smooth muscle of guinea-pig vas deferens following activation of α_1 -adrenoceptor stimulation by noradrenaline released from sympathetic axon terminals (Vizi et al, 1992). To determine the extent of ATP release from the detrusor muscle the ATP integral obtained from equivalent mean peak tension contractions evoked by electrical field stimulation and 3 μ M carbachol was compared. The ATP integral for EFS was significantly greater than

that for carbachol indicating that not all of the ATP signal could be attributed to the contraction of the muscle itself.

In the presence of 1 μ M atropine an ATP signal was recorded at all frequencies tested in a similar pattern to those of control. The addition of atropine reduced the EFS stimulated contractions and also the ATP integral. However atropine reduced the ATP signal to significantly lesser extent than tension. The reduction in ATP integral is probably due to a reduction in contraction and therefore a reduced ATP release from the activated muscle.

Following the addition of TTX no tension or ATP signal was recorded indicating that any ATP signal recorded is directly or indirectly related to neurotransmitter release from embedded nerves; from the nerve itself or from contraction of the muscle following neurotransmitter stimulation of appropriate receptors. The results indicate that a proportion of the ATP signal recorded during nerve-mediated stimulation is derived from the nerves.

4.9 FREQUENCY DEPENDENCE OF NEUROTRANSMITTER RELEASE

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The measurement of ATP signals following nerve-stimulation of detrusor smooth muscle from guinea-pig was used to investigate the frequency-dependence of neurotransmitter release, and in particular to determine if there was a preferential release of ATP at low stimulation frequencies.

The first evidence of frequency-dependent release were the 30 second integrals of ATP signals following nerve-stimulation over the frequency range 1 - 24 Hz. There were large increases in the integral with frequency from 1 to 12 Hz but any further increases in frequencies resulted in no additional increases in the integral. When the frequency required to produce half the maximal response ($f_{1/2}$) of tension and ATP integral were compared the ATP signal $f_{1/2}$ was significantly less.

The ATP integrals followed a similar pattern in the presence of atropine with a $f_{1/2}$ comparable to those in control conditions. This shows that the reduced contraction did not significantly affect the ATP signal and that a major proportion of the signal is due to constant levels of ATP released following stimulation of the nerves. Atropine did cause a shift in the $f_{1/2}$ of tension with atropine having a significantly greater effect at higher stimulation frequencies, indicting a greater importance of acetylcholine at these frequencies. In the presence of atropine when contraction is mediated by ATP only the tension and ATP integral followed a similar pattern of frequency dependence. They both reached a maximal response at equivalent frequency and there was no significant

difference in their $f_{1/2}$ value showing a direct relationship between release of ATP and contraction.

These combined results indicate that there is differential release of neurotransmitters during stimulation at various frequencies of the motor nerve in the detrusor smooth muscle. It is proposed that there is a preferential release of ATP at lower frequencies and acetylcholine at higher frequencies and this can be corroborated by previous studies with bladder tissue. In contrast to the atropine results in this study ABMA, the non-hydrolysable ATP analogue, caused a greater reduction of force at lower stimulation frequencies (Ikeda, 2006), again implying that a greater proportion of contraction is mediated by ATP at low frequencies. Similar findings using Ach and ABMA were made by Brading and Williams (1990). Also adenosine, NECA (a general A1/A2-agonist) and CPA (an A1-specific agonist) reduced the force of nerve-mediated contractions particularly at lower frequencies (Ikeda et al, 2006). As their effects may be due to altering transmitter release via A1-receptors, which preferentially reduces ATP release, this is consistent with preferential release of ATP at low frequencies of stimulation. Differential frequency-dependent release of neurotransmitters has also been described for other tissues (Todorov et al, 1999; Silinsky and Redman, 1996), as has the release of ATP at lower stimulation frequencies compared to its co-transmitter, NA (Kennedy *et al* 1986) and Ach (Vizi *et al* 1997). However this study provides direct evidence of preferential release of ATP at lower that has not previously been shown.

Although control experiments using direct muscle stimulation were used to estimate the proportion of ATP released from nerves and muscle it would be useful try to inhibit muscle contraction to rule out completely addition of ATP released from the muscle itself to the ATP signal measured. When the release of ATP from rat innervated hemidiaphragm was investigated at different frequencies of stimulation nicotinic receptor blockers D-tubocurarine and α -bungarotoxin were used to block nerve evoked contractions (Santos et al, 2003). The study showed this blockade inhibited over 60% of ATP released.

4.10 OVERALL CONCLUSIONS

ATP release following nerve stimulation acts as a co-transmitter with Ach to initiate contraction in detrusor smooth muscle in guinea-pig and some overactive but not stable detrusor from human bladders. There are three main factors that may account for the emergence of atropine resistant contractions in detrusor from overactive bladders; increased ATP release, reduced ATP breakdown and increased smooth muscle sensitivity. The latter option has previously been investigated and ruled out.

Tension experiments showed examples of how modulation of the activity of the ectonucleotidases can impact on the force of nerve-mediated contractions. Increased

ATP breakdown resulted in a reduction in force whilst decreasing activity augments force.

Gene expression studies revealed relative levels of E-NTPDase 1, 2, 3 and 5 in detrusor smooth muscle from stable and overactive human bladder samples. All four enzymes were expressed in both sample groups but the relative expression level of E-NTPDase 1 was substantially greater than the other three. These results indicate that alteration in ATP breakdown due to differences in enzyme expression could contribute to atropine resistance and hence overactivity. The cause for altered expression remains unknown.

Measuring ATP degradation rates showed that there was no difference in ectonucleotidase activity between detrusor from stable and overactive bladders. However experiments also showed that human bladder ectonucleotidase activity was similar to that of guinea-pig. Guinea-pig has the greatest ATP component of contraction compared to the human groups which have little or no atropine resistance, it is therefore hypothesised that more ATP is released from nerves in the guinea-pig to initiate contraction.

Experiments showed that a proportion of the signal measured with ATP sensitive electrodes was ATP released from stimulated nerves in the guinea-pig detrusor. ATP measured was dependent on frequency in the presence and absence of atropine. The purinergic component of contraction closely followed the integral of measured ATP, with maximal responses at equivalent frequencies and comparable $f_{1/2}$ values. The real-time measurement of ATP release with microelectrode biosensors provided evidence to

support frequency dependent release of neurotransmitters with preferential release of ATP at lower frequencies of stimulation.

Overall this thesis provides evidence for two of the hypothesis proposed to explain the emergence of atropine-resistant contractions in human bladder overactivity. Firstly expression levels of some ectonucleotidases were reduced which may lead to reduced breakdown of ATP in bladder pathology, particularly so as the highly expressed E-NTPDase 1 was reduced in overactive bladders. Secondly ATP degradation studies showed equivalent breakdown rates for ATP by ectonucleotidases in the three groups of tissues which had varying ATP components of contraction. It is possible therefore that atropine-resistance in overactive bladder is due to an increase in ATP released by nerve stimulation.

4.10 FURTHER RESEARCH

The role of ATP and its breakdown in detrusor contraction is important and may contribute to bladder overactivity. This study showed that by inhibiting ATP breakdown in guinea-pig contractile force can be increased. It would be interesting to investigate the effect of inhibiting ectonucleotidase activity in stable and overactive bladder samples and possibly to use new more selective inhibitors of these enzymes to establish the effects of the individual subtypes and if there were difference between human groups.

Although this study determined the relative expression levels of E-NTPDase 1, 2, 3 and 5 this does not give any indication of the level of protein actually present. Further molecular studies, including western blots, would provide further information on enzymes present and any possible differences between pathological groups. As would immuno-histochemical studies to investigate the localisation of the enzymes within the samples.

This study provided the first measurement of real-time release of ATP from guine-pig-detrusor smooth muscle with the use of novel ATP sensitive microelectrode biosensors. The protocol developed proved effective and could be applied to other tissues particularly human detrusor from stable and overactive bladders to establish if altered release of ATP from nerves contributes to atropine resistance in some overactive samples. The electrodes could also be useful in investigating the effect of pre-junctional modulators of ATP release including adenosine.

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